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A Cytopathologic Study of Tobacco Tar-Induced Lesions of Uterine Cervix of Mouse [1,2,3,4,5]

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SUMMARY

Neoplastic lesions were induced in the cervical and vaginal mucosae of C3H mice by applying, intravaginally, crude tobacco tar, 5 times weekly for 44 weeks, under strict visual control through an infant-size otic speculum. Weekly, during the period of carcinogenic treatment, vaginal smears of tobacco tar-treated mice, benzopyrene-treated mice, and untreated mice of the same strain were scored by 11 cytologic criteria of malignancy. Histologic evaluation of induced lesions was based upon three different sets of standards as follows: (1) Lesions were compared to benzopyrene-induced, early cervical carcinoma developed after a specified, gradually increasing number of weeks of benzopyrene treatment. (2) Lesions were classified by Suntzeff's grades for the extent of epithelial downgrowth. (3) Diagnoses were made by von Haam's classification of benzopyrene-induced cervical carcinoma. It was shown that cytologic findings and histologic lesions induced by tobacco tar in the cervix and/or vagina of C3H mice were strikingly similar to those observed during the development of 3,4-benzpyrene-induced (*Chemical Abstracts'* nomenclature: benzo[*a*]pyrene) cervical carcinoma. Since tobacco tar-induced lesions develop after prolonged treatment and persist for several months, their use may offer a better opportunity for morphologic and biologic studies of carcinoma *in situ* than that of rapidly growing neoplastic lesions induced by strong carcinogens, such as 3,4-benzpyrene or methylcholanthrene. Further study is necessary to determine the final stages of development of neoplastic lesions induced by tobacco tar. It was demonstrated that the uterine cervix is a suitable site for the study of carcinogenic properties of tobacco tar.—J. Nat. Cancer Inst. 23: 1–19, 1959.

NO DIRECT experimental evidence has been reported, as yet, to prove that tobacco tar induces irreversible changes in bronchial mucosa which would lead to progressive formation of bronchogenic carcinoma of the epidermoid type. There are two reasons why it is difficult to set up ex-

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[2] This investigation was supported in part by research grant C-2540 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] Data are based on a research investigation undertaken during the tenure of a Damon Runyon Memorial Fund Fellowship, 202(T).

[4] The experimental part of this investigation was carried out at the State University of New York, College of Medicine. The completion of this study was aided by a grant from the American Cancer Society, Inc., through the Institutional Research Grant at the Hahnemann Medical College, Philadelphia, Penna.

[5] A brief summary of the data was presented at the Sixth Annual Meeting of the Intersociety Cytology Council held in New York, November, 1958.

[6] The authors are indebted to Dr. A. I. Kosak, New York University, for a supply of tobacco tar, and to Dr. E. von Haam, for evaluation of histologic preparations submitted to him.

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periments for a direct evaluation of this question: (1) The lack of a simple technique which would permit numerous applications of the agent to a not readily accessible site. (2) Untreated, whole tobacco tar is highly toxic.

Malignant lesions were produced with crude tobacco tar after a long series of paintings of the skin (*1, 2*). However, these experiments were often curtailed because of the high toxicity of this agent. Histologically, the use of a mucous membrane rather than the skin was desirable to increase analogies with bronchial mucosa. Thus, the oral cavity became a target organ of choice by other investigators. Yet, no conclusions could be drawn from these experiments because of the premature loss of animals (*1, 3–6*).

More recently, squamous metaplasia was observed in biopsies after 11 months of direct application of tobacco tar to bronchial mucosa of dogs (*6*). In another pertinent study, mice were exposed to cigarette smoke, and cellular abnormalities, possibly representing carcinoma *in situ*, were noted in the bronchial mucosa in several of the animals after prolonged exposure to the agent (*7*).

In the present study, the uterine cervix of the mouse was used as a target organ for testing carcinogenic properties of tobacco tar because of the following considerations:

1. The cervix is readily accessible to repeated applications of tested chemicals and ideally suited for examinations of periodically collected cytologic material throughout the duration of the experiment (*8-12*).
2. Its small surface, requiring application of a minute amount of the agent to be tested, helps to minimize the toxic effects of tobacco tar.
3. Histologically, it is closer to the bronchial mucous membrane than the previously used site—the skin. Moreover, the animals cannot remove the deposited tar, which was possible when the buccal cavity was used in similar experiments (*4*).
4. The uterine cervix of the mouse is an extremely rare site for spontaneous carcinoma (*13–15*).

MATERIAL AND METHODS

Animals.—Female, virgin C3H mice, 4 to 6 weeks old, obtained from the Roscoe B. Jackson Memorial Laboratory, were caged in groups of 10 or less and given Purina laboratory chow and tap water *ad libitum*. Animals were kept at room temperature and transferred to clean cages once a week.

Tobacco tar.—Crude tobacco tar [its preparation was described elsewhere (*16*)] was stored in a brown bottle at −20° C. (*2*). A quantity sufficient for approximately 1 week of treatment was periodically removed from the stock sample by means of a glass rod that was attached to the screw-type stopper of a small medicine bottle. After daily use the glass rod, with the remaining amount of tar, was placed in the bottle and kept in the freezing compartment of a refrigerator (*2*).

The whole, unaltered, tobacco tar was applied by a wire-loop applicator to the uterine cervix of mice, 5 times weekly. The applicator was inserted under visual control through an infant-size otic speculum—#1 for young mice and #2 as the mice grew bigger (*9*). A minute quantity, corresponding to about 0.15 mg., was placed on the cervix. In addition, in the hope that this might facilitate and/or prolong the contact of the agent with the tissues, a thread was inserted into the endocervical canal of the mice, by Murphy's technique (*17*).

Benzopyrene.—3,4-benzpyrene [7] was obtained from Edcan Laboratories. A 1 percent solution in acetone was applied twice weekly under visual control and through an otic speculum. Details of this technique were reported elsewhere (*18*).

Evaluation of Findings

Cytology.—Weekly, vaginal smears, prepared as described in an earlier communication (*9*), were stained by Papanicolaou's method with EA50. Smears were scored by 11 arbitrarily established cytologic criteria of malignancy [nuclear enlargement, hyperchromasia, irregular nuclear borders, prominent nucleoli, binucleation and multinucleation, cytoplasmic areas of basophilia, cytoplasmic vacuolization, bizarre clusters, elongated cells, engulfment, and keratinization (*9*)]. The criterion for each smear was: absence (−); doubtful presence (±); definite presence (+); and striking frequency (++). Based on results of previous experience, repeated scores of at least 5 criteria (+) were accepted as suggestive of a malignant neoplasm or, if strikingly positive (++), as conclusive for a malignant neoplasm. Scores of 3 to 5 involvements (± and +) were considered as borderline atypical findings and those below 3 as negative.

Inspection of cervix.—At the time of application of the agent to be tested, the cervix and vagina of each mouse were inspected for bleeding and/or grossly visible lesions. Cervices of untreated control mice were also examined occasionally.

Pathology.—Dead mice were autopsied routinely. The selection of organs for histologic examination depended on gross autopsy findings. As a rule, however, all uteri were fixed in 10 percent formalin and then cut lengthwise anteroposteriorly to retain the topographical relation of the bladder and rectum and to show cervical and vaginal tissues in the same section (*18*). Sections were cut and stained with hematoxylin and eosin. The histologic findings in the uteri of tobacco tar-treated animals were compared with benzopyrene-induced lesions found at various stages of development of cervical carcinoma, and were also classified by the VC grades of Suntzeff *et al.*, for the description of estrogen-induced carcinoma-like lesions in the vagina and cervix of the mouse (*19*). In addition, the tobacco tar-induced lesions were diagnosed according to von

[7] *Chemical Abstracts'* nomenclature: benzo[a]pyrene.

Haam's classification (*8, 12*) when histologic preparations from 16 representative animals were submitted for his opinion.

Experimental Study

Tobacco tar treatment was applied to 30 mice. Twenty of these were treated without, and 10 with, a thread inserted intracervically. Since no difference attributable to this intracervically inserted thread was observed among tobacco tar-induced lesions, thread as a factor has been disregarded in subsequent considerations. After 44 weeks of painting, the mice were killed.

Benzopyrene treatment was applied to 50 mice. These were divided into 2 groups. In one group of 20 mice, treatment was continued until they were killed: 2 mice every other week beginning on the 3d through the 21st week of benzopyrene painting. In the second group, 30 mice were treated for 19 weeks and then observed for variable lengths of time up to 11 weeks.

No treatment was applied to 5 control mice, which were killed after 46 to 58 weeks of observation.

RESULTS

Tobacco Tar-Treated Mice

Cytologic findings were considered strongly suggestive of an early carcinoma (+) in 19 mice, borderline atypical (±) in 10, atypical but not suspicious in 1. Eleven of the 30 tobacco tar-treated mice developed suspicious borderline scores by the 16th and 20th week of treatment and 1 mouse as early as the 12th week. In most of the mice, cytologic findings became definitely suggestive of an early malignant neoplasm after approximately 30 to 34 weeks of treatment, either as a primary observation or after previous transitory, somewhat suspicious, findings. The most striking cytologic scores were noted after 38 to 39 weeks of treatment in 8 of 30 mice.

Cytoplasmic vacuolization, presence of elongated cells, and engulfment were striking in tobacco-tar lesions. Keratinization and basophilic cytoplasmic areas were perhaps the least pronounced among the recorded criteria. Examples of cells, which meet certain cytologic criteria of malignancy as compared to cells found in vaginal smears of untreated controls and of mice treated with 3,4-benzpyrene, are illustrated in figures 1 through 12. The summary of the cytologic evaluation in individual mice is compared to corresponding histologic findings in table 1. In addition, the average number of cytologic criteria determined per mouse per week, together with the corresponding curves for the benzopyrene-treated group and control mice, are shown in text-figure 1.

A comparison with 3,4-benzpyrene-induced cervical carcinomas shows

TABLE 1.—Cytologic findings and histologic evaluation of tobacco tar-induced lesions in the cervix and/or vagina of C3H mice

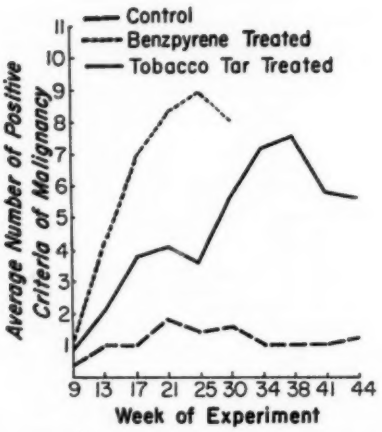
Mouse No.	Cytologic findings	Histologic findings according to: von Haam, E.	Grades of* Suntzeff *et al.*	Similar lesions seen in mice treated with benzopyrene for (*weeks*):
1	±	Dysplasia	1+	11
2	±	Carcinoma *in situ*†	2+ or 3	13–15
3	±	Carcinoma *in situ*	3 or 4	17
4	±	Dysplasia with carcinoma *in situ*	1 or 2	11
5	±	Carcinoma *in situ* with microinvasion	3+	15
6	±	Dysplasia with carcinoma *in situ*	1 or 2	11
7	+	Dysplasia with extensive carcinoma *in situ*	2	13
8	+	Carcinoma *in situ* with microinvasion†	3	15–17
9	±	Carcinoma *in situ*	2 or 3	13
10	±	Carcinoma *in situ* with microinvasion	3	15–17
11	±	Early invasive carcinoma	4	17
12	+	Carcinoma *in situ* with microinvasion†	3	15–17
13	±	Carcinoma *in situ* (isolated)	3	15
14	±	Carcinoma *in situ* with microinvasion†	3	15–17
15	+	Invasive carcinoma†	4	17
16	+	Carcinoma *in situ* with microinvasion†	3 or 4	15–17
17	+	Dysplasia with carcinoma *in situ*	2 or 3	15
18	+	Invasive carcinoma	4	17
19	+	Carcinoma *in situ*†	3	15
20	+	Invasive carcinoma†	3 or 4	17
21‡	+	Invasive carcinoma†	4	17
22	−	Dysplasia†	1 or 2	11
23	+	Invasive carcinoma	4	17
24	+	Carcinoma *in situ* with microinvasion†	3 or 4	15–17
25	+	Carcinoma *in situ*†	2+	15
26	+	Dysplasia with carcinoma *in situ*†	2+ or 3	13–15
27	+	Carcinoma *in situ* with microinvasion†	3 or 4	17
28	+	Early invasive carcinoma†	3 or 4	17
29	+	Early invasive carcinoma†	3 or 4	17
30	+	Invasive carcinoma	4	17

*Grade 0 = absence of downgrowth or presence of only very short epithelial processes.
Grade 1 = epithelial processes extending for a moderate distance into the underlying tissue.
Grade 2 = epithelial processes which reach to a considerable depth and which may, in addition, have begun to branch out.
Grade 3 = precancerous downgrowth, extending deeply and invading connective tissues irregularly with branching processes, frequently showing hyaline pearls.
Grade 4 = marked cancer-like invasion of the underlying connective tissue and adjoining parts.
†Diagnosed by von Haam.
‡Last 10 mice had thread inserted into cervical canal.

that cellular abnormalities were somewhat less pronounced and appeared later in the tobacco-treated mice than in the benzopyrene-treated mice. Also, their development was slow, the stationary periods were prolonged, and temporary regressions were observed. With optimal doses of benzopyrene (biweekly paintings through a speculum for 19 weeks), the progress of cytologic scores after the 17th week of treatment was very rapid (text-fig. 1).

Inspection of the Cervix During Treatment

Seventeen of 30 mice had small bleeding spots and/or erythematous areas on the cervix or vagina. These signs were unrelated to the presence of the inserted thread and there was no consistency in the time of appear-



TEXT-FIGURE 1.—Average number of cytologic criteria of malignancy, determined per mouse per week, are shown for comparison of tobacco tar- and benzopyrene-treated and control mice. The interval between consecutive dates at which averages were obtained was 4 weeks, with a few exceptions made because of scantiness of smears.

ance of such findings. Furthermore, the thread was rarely seen in the cervix after the initial phase of the experiment. Apparently all threads were lost since none were recovered at autopsy.

Pathologic Findings

At autopsy, the killed animals did not show the grossly characteristic tumors replacing the uterus. These tumors, which were seen in benzopyrene- or methylcholanthrene-treated mice, have previously been described in detail by various authors (*8–12*, *17*). In general, the uteri appeared grossly normal except that in several mice granularity of the uterine portio and minute, ill-defined, vaginal nodules were noted when cutting the fixed tissues. Hydrosalpinx was found in 2 of these mice. Mammary carcinoma was found in 7 of the 30, but there appeared to be no correlation between the appearance of cytologic scores suggestive of cervical neoplasia and the presence or absence of mammary carcinoma.

Histologic Examination

Neoplastic changes in various stages were seen. These changes are compared to lesions produced in 3,4-benzpyrene-treated mice and to normal vaginal mucosa of a control mouse, and are illustrated in figures 13 through 24. VC grades and diagnoses, according to von Haam, in individual mice are presented and correlated with cytologic findings in table 1. Table 1 also shows that the induced lesions fall mostly into Suntzeff's VC-2 through VC-4 categories, with a predominance of the VC-3 grade. Most of the induced lesions represent intra-epithelial

carcinoma, either with or without some evidence of invasion, as judged by von Haam's classification.

Benzopyrene-Treated Mice

In a group of 30 mice painted twice a week for 19 weeks with 3,4-benzpyrene, under visual control through a speculum, all had invasive carcinoma within 19 to 30 weeks of the experiment. Cytologic findings in these mice compared to tobacco tar-painted mice are shown in text-figure 1, and were reported in detail elsewhere (*18*).

In a group of mice painted with 3,4-benzpyrene, with 2 mice killed every other week, a wide range of histologic lesions was represented and their progressive nature was apparent. Histologic findings in these mice were considered representative of the developmental stages of induced cervical carcinoma and compared to tobacco tar-induced lesions (table 1 and figs. 14 through 24).

By the 18th week of treatment, mice usually began to bleed and inspection of the cervix revealed a neoplastic growth.

Untreated Control Mice

No demonstrable neoplastic lesions developed in untreated mice. Cytologic findings in these mice are shown in text-figure 1.

DISCUSSION

The observations reported here do not warrant definite conclusions on the ultimate outcome of tobacco tar-produced lesions, since animals were killed after 44 weeks of treatment. It may be significant that these lesions were strikingly similar to early developmental stages of benzopyrene-induced cervical carcinoma. In 17 of 30 tobacco tar-treated mice, such lesions corresponded to early carcinoma that was produced after about 17 weeks of benzopyrene treatment. It has been reported elsewhere that vaginal smears of C3H mice become suggestive of an early carcinoma on or about the 17th week of benzopyrene treatment. It was demonstrated in the same communication that benzopyrene-treated mice died of cervical carcinoma in approximately 11 weeks after 19 weeks of treatment (*18*). Thus, by keeping tobacco tar-treated mice under observation for several months longer, one probably could determine whether produced lesions would ultimately develop a malignant course. Such experiments are now in progress.

It has been demonstrated in the course of this study that the toxicity factor of tobacco tar may be practically disregarded when the uterine cervix is used as a target organ. There was not a single casualty among

the series of 30 mice, despite the fact that each mouse received approximately 220 applications of tobacco tar.

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PLATES

PLATE 1

Cells in vaginal smears. × 1,250

FIGURE 1.—Normal epithelial cells. Untreated control mouse #31, after 17 weeks of observation.

FIGURE 2.—Abnormal cell exhibiting multinucleation, after 39 weeks of tobacco-tar treatment. Mouse #12.

FIGURE 3.—Binucleation. Mouse #19, after 41 weeks of tobacco-tar treatment. *Compare* with figure 4.

FIGURE 4.—Binucleation. Mouse #33, after 12 weeks of benzopyrene treatment. Tissues of this mouse illustrated in figure 18.

FIGURE 5.—Bizarre cluster with engulfment. Mouse #23, treated with tobacco tar 40 weeks. Cervical carcinoma induced in this mouse is illustrated in figures 19 and 20. *Compare* with figure 6.

FIGURE 6.—Bizarre cluster with engulfment. Mouse #32, treated with benzopyrene, 12 weeks.

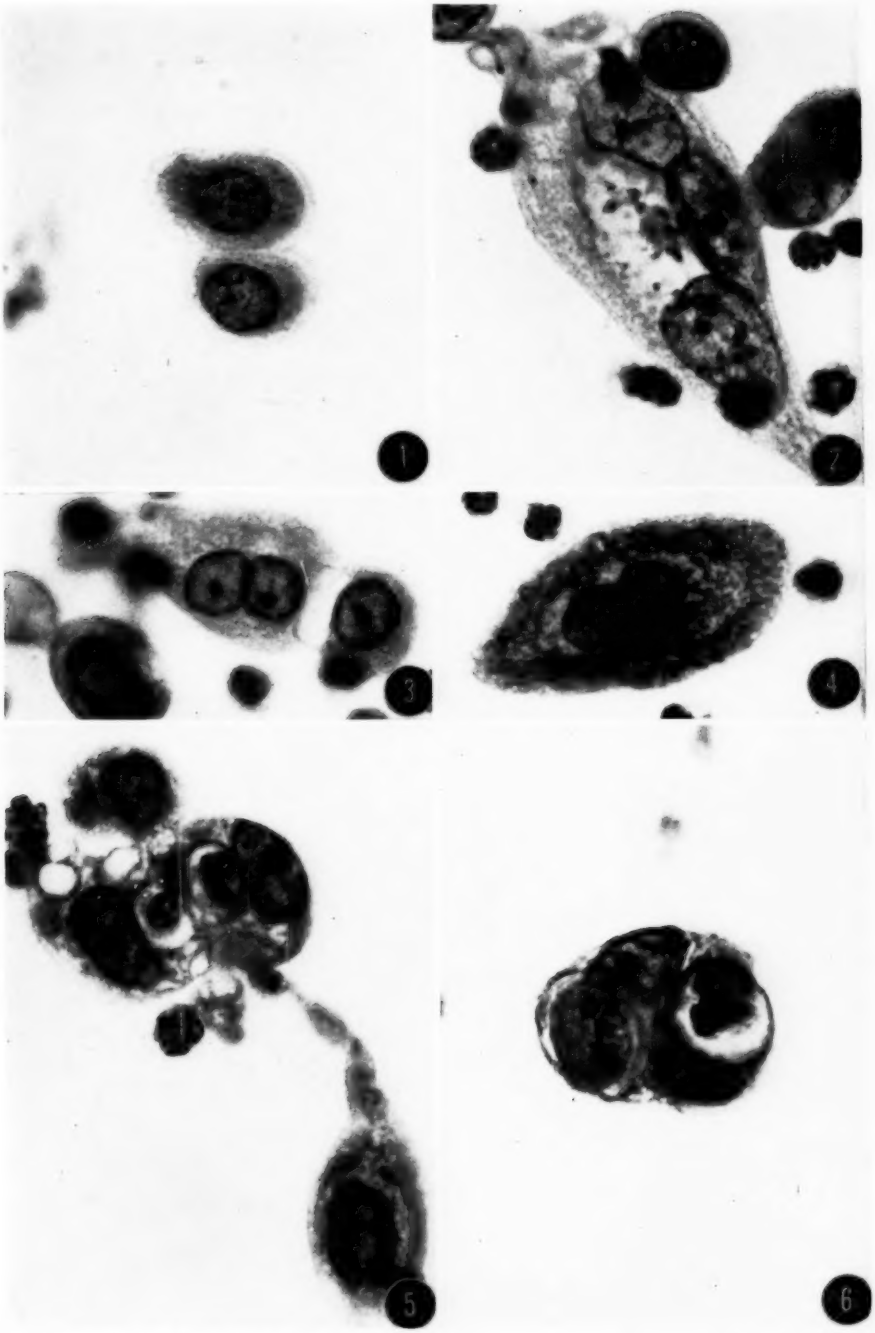

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PLATE 2

Cells in vaginal smears. × 1,250

FIGURE 7.—Cluster with vacuolation. Mouse #15, after 23 weeks of tobacco-tar treatment. Tissues of mouse #15 illustrated in figures 23 and 24. *Compare* with figure 8.

FIGURE 8.—Cluster with vacuolation. Mouse #32, after 12 weeks of benzopyrene treatment.

FIGURE 9.—Localized basophilic cytoplasmic area. Mouse #15, after 13 weeks of tobacco-tar treatment. *Compare* with figure 10.

FIGURE 10.—Localized basophilic cytoplasmic area. Mouse #34, after 12 weeks of benzopyrene treatment.

FIGURE 11.—Bizarre elongated cell. Mouse #15, after 23 weeks of tobacco-tar treatment. *Compare* with figure 12.

FIGURE 12.—Bizarre elongated cell. Mouse #32, after 12 weeks of benzopyrene treatment.

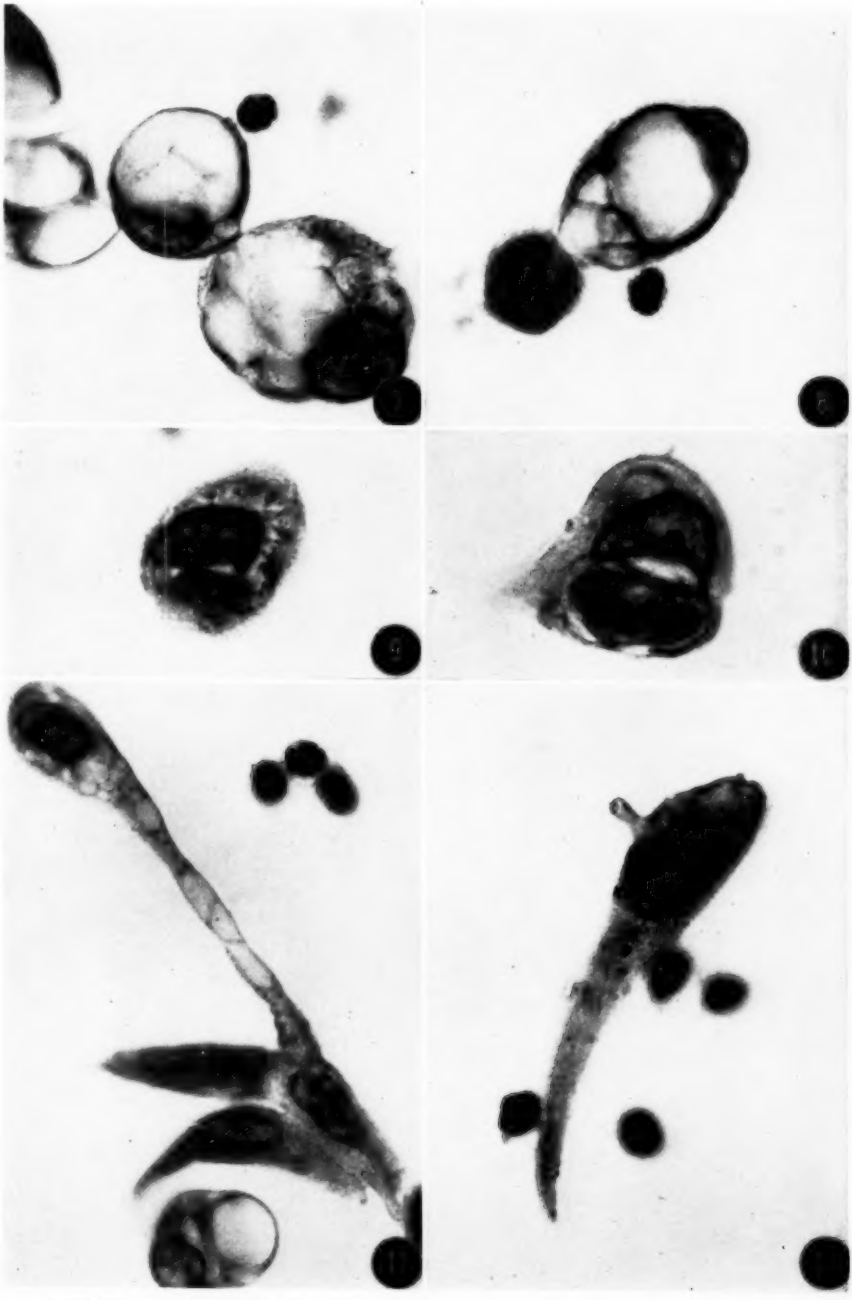

PLATE 3

FIGURE 13.—Section of normal vaginal mucosa of untreated control mouse #31, × 125. *Compare* with figure 14. (Cells in vaginal smear of same mouse are shown in fig. 1.)

FIGURE 14.—Section of cervix and vaginal mucosa of tobacco tar-treated mouse #7, showing dysplasia (A) and carcinoma *in situ* (B). × 125

FIGURE 15.—Dysplasia. Same section shown in figure 14. × 500. *Compare* with figure 16.

FIGURE 16.—Carcinoma *in situ*. Same section shown in figure 14. × 500

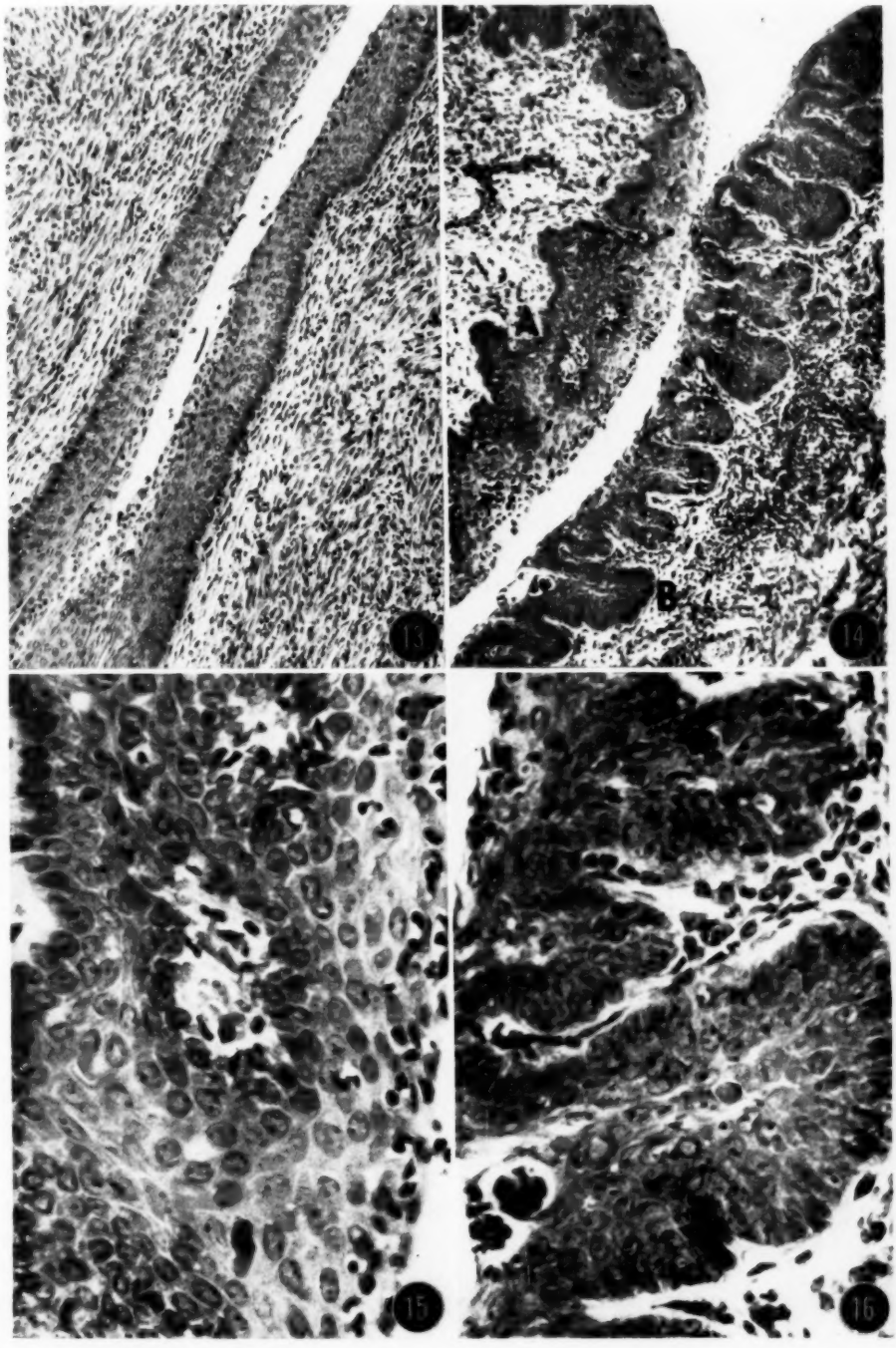
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PLATE 3

FIGURE 13. Section of normal vaginal mucosa of untreated control mouse #31. × 125. *Compare* with figure 14. (Cells in vaginal smear of same mouse are shown in fig. 1.)

FIGURE 14. Section of cervix and vaginal mucosa of tobacco tar-treated mouse #7, showing dysplasia (A) and carcinoma *in situ* (B). × 125

FIGURE 15. Dysplasia. Same section shown in figure 14. × 500. *Compare* with figure 16.

FIGURE 16.—Carcinoma *in situ*. Same section shown in figure 14. × 500

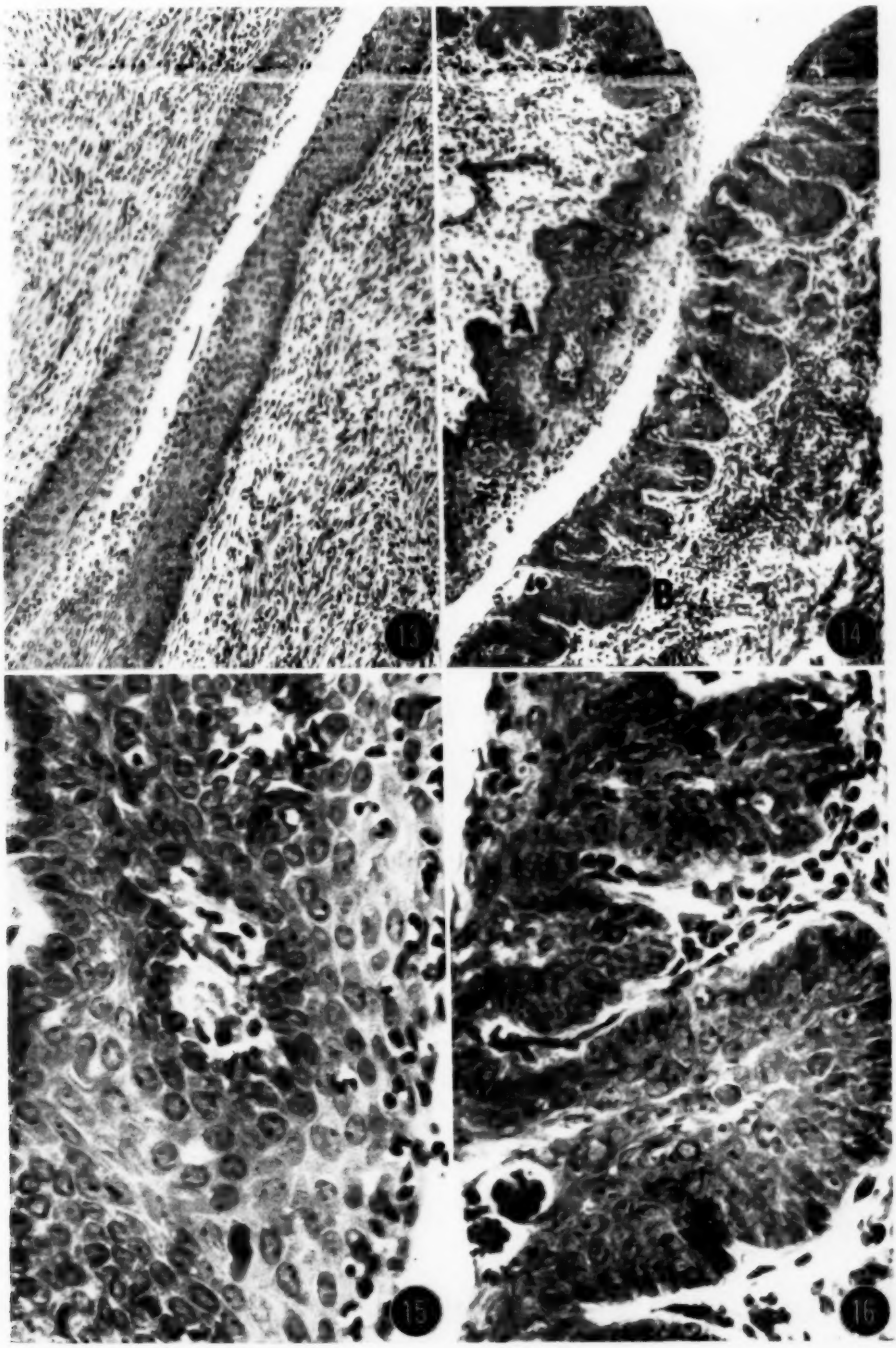
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PLATE 4

FIGURE 17.—Carcinoma *in situ* with microinvasion, cervix, tobacco-treated mouse #14. × 125. *Compare* with figure 18.

FIGURE 18.—Carcinoma *in situ* with microinvasion, cervix, mouse #33, after 12 weeks of benzopyrene treatment. × 50

FIGURE 19.—Epidermoid-cell carcinoma, invasive, cervix, tobacco-treated mouse #23. × 50. For illustration of smears of this mouse, *see* figure 5.

FIGURE 20.—Same section as that in figure 19. × 125. *Compare* these sections of tobacco tar-treated mouse with those of benzopyrene-treated mouse #35, in figures 21 and 22.

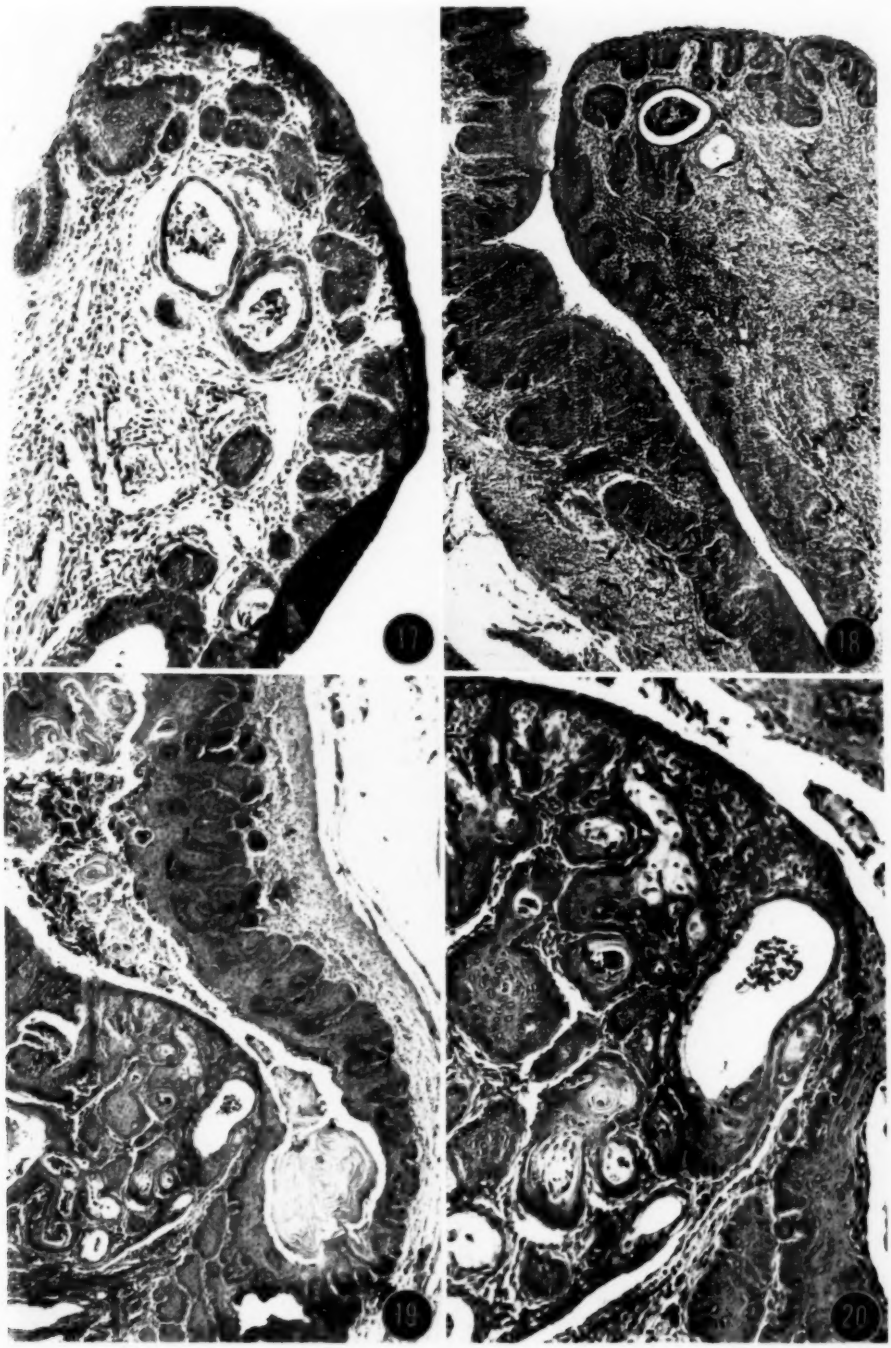
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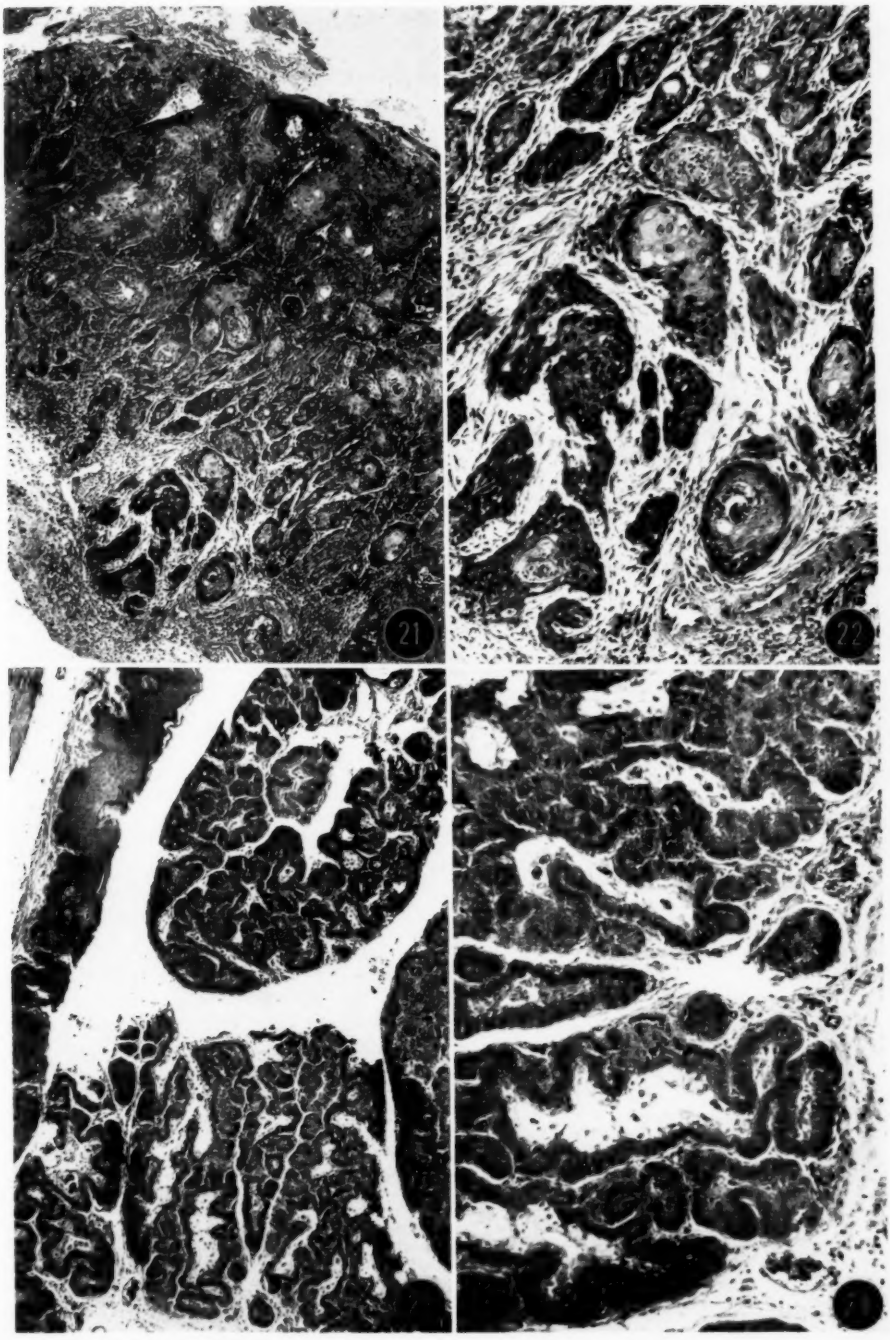
PLATE 5

FIGURE 21.—Epidermoid-cell carcinoma, invasive, cervix, mouse #35, treated for 19 weeks with benzopyrene. × 50

FIGURE 22.—Same section shown in figure 21. × 125

FIGURE 23.—Carcinoma, cervix, muco-epidermoid variety; tobacco-treated mouse #15. × 50. (For cellular findings in this mouse, *see* figs. 7, 9, and 11.)

FIGURE 24.—Same section shown in figure 23. × 125



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Correlation Between Yoshida Sarcoma and Human Neoplastic Disease In Cancer Chemotherapy Studies [1,2,3]

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SUMMARY

Experimental and clinical cancer chemotherapy studies with Yoshida sarcoma in rats and in 61 inoperable or advanced cancer patients are reported. The chemicals used were colchicine derivatives, *p*-phenylenediphosphoric acid tetraethyleneimid (RC4), and 1-thia-3-azaazulan-2-one (Az.). In these studies, combination chemotherapy with RC4 and Az. showed the best results. The combined use of 2 substances which have entirely different modes of action prevented growth of resistant tumor cells, and effective treatment by a small amount of long-term continued injections on tumor cells was substantiated. A number of patients with carcinoma of the breast, lung, and digestive tract showed objective or subjective improvement by the combination chemotherapy. If properly used, chemotherapy appears to have merit as an additive method in conjunction with surgical intervention in the treatment of certain types of inoperable and metastatic cancer, though a high rate of long-term survivors was not obtained with the present combination chemotherapy.—J. Nat. Cancer Inst. 23: 21–51, 1959.

MANY ANTICANCER chemicals, antibiotics, and physical agents have shown some efficacy in the treatment of human cancer. However, these substances were not effective on Yoshida sarcoma, which is in a state of pure culture about 4 or 5 days after transplantation. In 1951, Ishidate and Yoshida reported that 40 percent of rats with Yoshida sarcoma were cured completely by repeated intraperitoneal injections of nitrogen mustard, *N*-oxide (Nitromin), but the effects of the subcutaneously repeated injections were not clearly described (*1*). Recently, we found that 73 percent of rats with Yoshida sarcoma in pure culture were cured by repeated subcutaneous injections of *p*-phenylenediphosphoric acid tetra-

[1] Received for publication October 13, 1958.

[2] This investigation was supported by a grant-in-aid for scientific research from the Ministry of Education in Japan.

[3] Presented in part at the symposium on the Chemotherapy of Cancer for the Pacific-Asian Area and joint meeting with Cancer Chemotherapy Committee in Japan, October 24 to 28, 1957, Tokyo, Japan, and published as an abstract in the Scientific Proceedings of that meeting.

[4] The authors express their deep gratitude to Prof. T. Nozoe, Prof. S. Seto, Asst. Prof. Y. Kitahara, and their colleagues in the Tohoku University, Sendai, Japan, for the supply of a series of compounds with unsaturated 7-membered ring structure. We also take this opportunity to acknowledge with thanks the supply of RC4 given by the Takamine Laboratory, Sankyo Company, Tokyo, Japan. We are indebted to Professor Akazaki and his associates in the Pathological Department, Tohoku University, Sendai, Japan, and to Dr. N. Matoba, a surgical pathologist in our department, for their generous help and advice.

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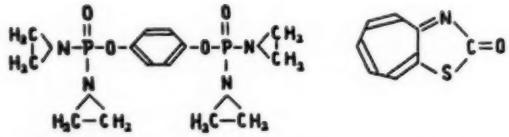
ethyleneimid (RC4) [5] combined with 1-thia-3-azaazulan-2-one (Az.) [5] (text-fig. 1).

Dewar, in 1945, proposed an unsaturated 7-membered ring structure for colchicine (*2*). In 1950 Nozoe *et al.* reported the synthesis of the mother substance, tropolone (*3*). Since then we have studied the anticancer action of these compounds, which have the unsaturated 7-membered ring structure, by using the Yoshida sarcoma (*4–11*). The compounds were divided into four categories: monocyclic tropoids, bicyclic tropoids, colchicine derivatives, and azulenoids.

In 1956, Miura examined the effect of azaazulenes on the incorporation of P^{32} in the polyribonucleotides of rat liver (*12, 13*). The results were as follows: 6-Mercaptopurine and 8-azaguanine inhibited the P^{32} incorporation with the polyribonucleotides of rat liver, at concentrations of 10^{-3} M and 10^{-4} M, 20 and 18 percent in the former and 50 and 15 percent in the latter, respectively. Az. inhibited the incorporation at concentrations of 10^{-3} M, 10^{-4} M, and 10^{-5} M, 69, 57, and 41 percent, respectively. The acute toxicity of these 3 compounds was similar. Az. seemed to possess a potential activity. However, prolongation of the survival



TEXT-FIGURE 1.—Structure of *p*-phenylenediphosphoric acid tetraethyleneimid and 1-thia-3-azaazulan-2-one.

period and cytologically noticeable changes were not observed in Yoshida sarcoma by repeated administration of this substance alone. This was true also for the rats treated with 6-mercaptopurine or 8-azaguanine.

We examined RC4, synthesized in the Takamine Laboratory in Japan (*10, 14*), and found that the anticancer action of RC4 on Yoshida sarcoma was similar to that of TEM, TEPA, and thio-TEPA, but was much less toxic than these compounds. However, the tumor cells were not controlled by the successive subcutaneous injection of RC4 alone.

The failure of many experiments with Yoshida sarcoma may be due to the following reasons: 1) The effective coefficient of the experimental substances is not sufficiently high. 2) Despite occasional temporary remissions, the tumor cell develops resistance to the therapy.

In order to clarify these points, the combined use of two chemicals, each of which has an entirely different mode of action, was attempted in the experimental chemotherapy. As a result of the experiment a higher ratio of animals was cured.

These findings led us to investigate further the possible action of combination chemotherapy with RC4 and Az. in patients with inoperable or

[5] *Chemical Abstracts'* nomenclature: *p*-phenylene(1-aziridinyl)phosphinate; 2*H*-cycloheptathiazol-2-one.

far-advanced cancer. This report is concerned with the results obtained from a therapeutic trial of these drugs in conjunction with our experimental and clinical investigations.

EXPERIMENTAL STUDY

Materials and Methods

A noninbred strain of Japanese rats was used in this study. The Yoshida sarcoma was transplanted weekly, according to the method of Yoshida (*15*). The abdominal wall was punctured with a sharp-pointed, sterile glass pipette (drawn from a 4-mm. outside-diameter glass tubing). In this way, sufficient tumor ascites for observation or transplantation was easily obtained. Ascitic fluid, 0.02 to 0.1 cc., containing about 20 to 100 million tumor cells was transplanted to each of a group of rats that weighed 100 to 150 gm. After inoculation, fluid was withdrawn each day from the peritoneal cavity, and proliferation of tumor cells in fresh and Giemsa-stained smears was observed until the death of the animal. Almost all rats died within 12 days after inoculation. Four or 5 days after inoculation there was a pure culture of tumor cells and the experimental chemotherapy was initiated. Rats showing spontaneous regression were discarded. In this report all negative data are excluded.

Effect of Single Injection With Colchicine Derivatives

Akaishi (*16*) studied the anticancer action of several colchicine derivatives. In table 1, data are presented on our work with 7 colchicine derivatives. The minimum effective dose (MED) here is defined as the dose given in a single subcutaneous injection which induces abnormal metaphase accumulation and disappearance of anaphase and telophase for up to 12 hours. If the effective coefficient is defined as the minimum

TABLE 1.—Effect of single subcutaneous injection of colchicine derivatives on Yoshida sarcoma

Derivatives	MLD/MED				
	3 hours	6 hours	12 hours	24 hours	48 hours
Colchicine	2	2	2	—	—
TMCA*	50	25	10	2.5	—
Thiocolchicine	10	10	5	5	—
Thiocolchiceine	2	2	2	—	—
Condensation product of colchiceine hydrazide with acetone (CPA)	6	6	3	1.5	1.5
Condensation product of colchiceine hydrazide with methyl ethyl ketone (CPM)	1	1	1	—	—
Colchiceinamide	20	10	4	—	—

*Trimethylcolchicinic acid methyl ether-D-tartrate.

lethal dose (MLD) divided by the MED, all but 1 of the 7 chemicals had an effective coefficient of over 1 at 6 hours. However, this ratio decreased with time. If this effect is considered up to 24 hours, there are 3 substances, trimethylcolchicinic acid methyl ether-D-tartrate (TMCA), thiocolchicine, and a condensation product of colchiceine hydrazide with acetone (CPA), of which the MLD/MED at 24 hours is 2.5, 5, and 1.5, respectively.

Repeated Administration of Thiocolchicine and Other Derivatives

The most effective dose for the repeated intraperitoneal administration of thiocolchicine was 1 to 0.5 mg. per kg. (1/5–1/10 MLD) every 12 hours. When repeated administration of thiocolchicine was started at the 4th day after transplantation, the metaphase accumulation reached its maximum at 60 hours after injection, while scattered diplochromosomes were most numerous at 48 hours. Necrobiotic and necrotic pictures of resting cells became noticeable at 48 hours, and a fairly large number of giant cells appeared after 72 hours accompanied by active phagocytic cells, such as leukocytes and monocytes. Telophase was observed, however, in a few cells at this time. It was impossible to destroy all of them by further administration of the compound.

In comparison with the controls, the survival period of the animals was prolonged. The results with other derivatives of colchicine listed in table 1 were similar to those obtained with thiocolchicine.

Effect of Single Injection With Several Alkylating Agents

The data presented summarize results of experiments performed, during a 2-year period, with over 2,000 animals. The results of these studies will be discussed elsewhere in greater detail (*17*) and only representative experiments are given here.

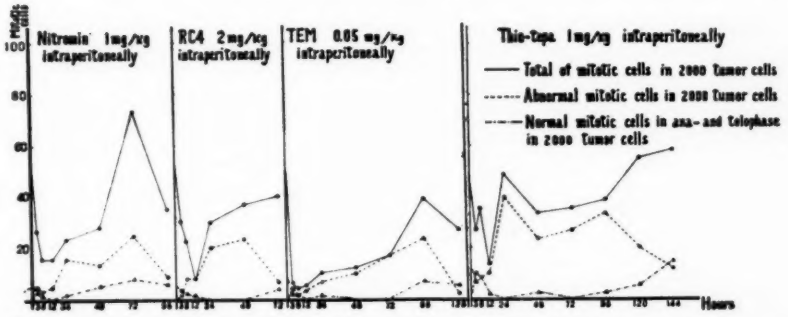
The minimum effective dose (MED) is defined as the dose necessary to induce abnormal mitoses and disappearance of anaphase and telophase.

Text-figure 2 shows the MED with a single intraperitoneal injection of Nitromin, RC4, TEM, and thio-TEPA.

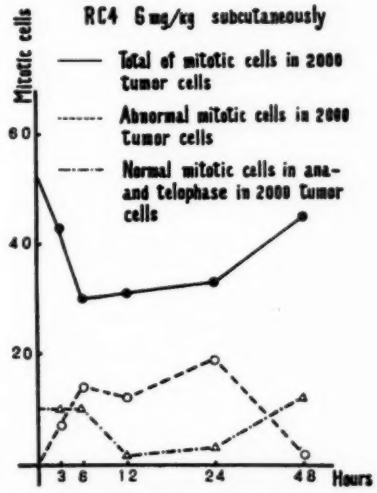
The abnormal mitoses include scattering and coagulation of chromosomes in metaphase, chromosome-bridge formation, and delayed chromosomes in anaphase and telophase.

TABLE 2.—The MED of Nitromin, RC4, TEM, and thio-TEPA given as a single intraperitoneal injection to rats with Yoshida sarcoma

Compound	LD50 (*mg./kg.*)	MTD (*mg./kg.*)	MED (*mg./kg.*)
Nitromin.....	80	40	1. 0
RC4.....	120	80	2. 0
TEM.....	3	1	0. 05
Thio-TEPA...	7. 5	5	1. 0


Nitromin 1 mg/kg intraperitoneally
RC4 2 mg/kg intraperitoneally
TEM 0.05 mg/kg intraperitoneally
Thio-tepa 1 mg/kg intraperitoneally
Total of mitotic cells in 2000 tumor cells
Abnormal mitotic cells in 2000 tumor cells
Normal mitotic cells in ana- and telophase in 2000 tumor cells
Hours

TEXT-FIGURE 2.—Mitotic rate in Yoshida sarcoma cells after a single intraperitoneal injection of 1 mg. per kg. of Nitromin, 2 mg. per kg. of RC4, 0.05 mg. per kg. of TEM, and 1 mg. per kg. of thio-TEPA. Each point represents an individual rat in which 2,000 cells were counted.


RC4 6 mg/kg subcutaneously
Mitotic cells
Total of mitotic cells in 2000 tumor cells
Abnormal mitotic cells in 2000 tumor cells
Normal mitotic cells in ana- and telophase in 2000 tumor cells
60
40
20
3 6 12 24 48 Hours

TEXT-FIGURE 3.—Mitotic rate in Yoshida sarcoma cells after a single subcutaneous injection of 6 mg. per kg. of RC4. Each point represents an individual rat in which 2,000 cells were counted.

Table 2 gives the MED of these substances tested intraperitoneally in rats with Yoshida sarcoma. The LD50 and the maximum tolerated dose (MTD) in non-tumor-bearing rats are also shown.

Text-figure 3 illustrates the effect of a single subcutaneous injection of 6 mg. per kg. of RC4.

Multiple Treatment With RC4, Az., and TMCA, Alone and in Combination

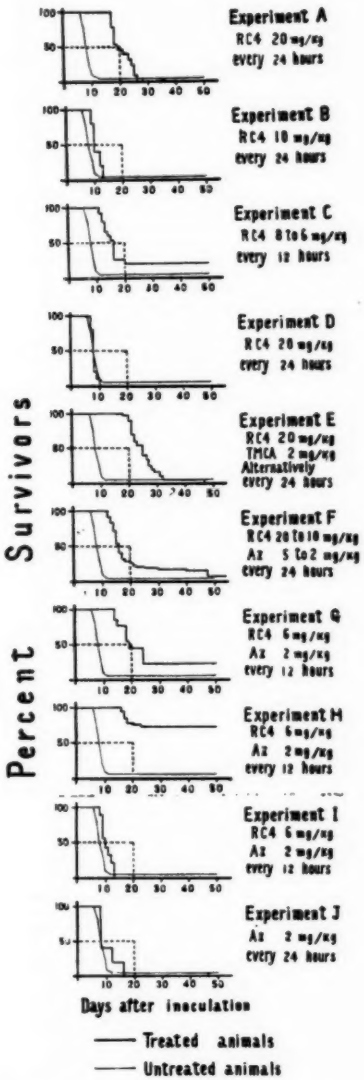
The influence on advanced Yoshida sarcoma of 10 types of multiple-treatment schedules, alone and in combination, was investigated. Twenty

cc. of physiological saline solution, 20 mg. of glucuronic acid, and 20 mg. of vitamin K per kg. of body weight, respectively, were given daily to all animals treated subcutaneously. The average survival time of 172 of 182 control animals without treatment was 8.2 days. Spontaneous regression of tumor cells occurred in 10 animals, that is, 5.5 percent of 182 control animals. The LD50 and MTD in non-tumor-bearing rats receiving injections of Az. were about 150 and 100 mg. per kg. of body weight, respectively. The treatment schedules and the results are shown in table 3 and text-figure 4.

Repeated subcutaneous injection of RC4, 20 mg. per kg. (expt. A, table 3, and text-fig. 4).—With the repeated subcutaneous injection of RC4, 20 mg. per kg., the average survival period was 20.5 days. The animal's life was prolonged 2.5 times that of the control. However, no animal was cured. The amount of ascites started to decrease from the 3d day after injection and became clearer. Four to 5 days after injection, the tumor that grew at the puncture site of the abdominal wall decreased in size to a scar. One week later, only small amounts of ascites could be obtained, but 10 to 12 days after injection the ascites started to reaccumulate, accompanied by an increase of tumor cells. Solid tumor became palpable intra-abdominally and the animals eventually died. Morphologically, cells with normal mitosis noticeably decreased from day to day. Cells in anaphase and telophase disappeared on the 3d day. The number of abnormal mitotic cells rose to a maximum on the 3d and 4th days and continued to be present. Four to 5 days after injection many monocytes were observed, among which irregular tumor cells were scattered. Microscopic examination revealed a few pyknotic cells about 1 week after injection. Ten to 12 days after injection, however, monocytes and leukocytes disappeared, while tumor cells reappeared and increased rapidly during 2 to 3 days so that the ascitic fluid again appeared to be a pure culture. The animals died even if a series of continued injections of the drug were given. There was not much change in body weight during the entire period of observation.

Repeated subcutaneous injection of RC4 every 12 hours (expt. C, table 3, and text-fig. 4).—The first dose was 20 to 10 mg., the second, 10 to 8 mg., the third, 8 to 6 mg., and the maintenance dose was 8 to 6 mg. per kg. of body weight. The body weight of the experimental animals was not decreased with this dosage schedule. Side effects could also be controlled. However, only 3 of 15 rats survived for a long time. The rest of the animals died of tumor before 30 injections were given. The course during treatment did not differ much from that described in the experiment with subcutaneous injections of RC4 given every 24 hours. From the 9th to 13th day after treatment, regrowth of tumor cells could not be controlled and the animals died.

Repeated injection with RC4 and TMCA alternately (expt. E, table 3, and text-fig. 4).—An initial injection of 4 mg. of TMCA followed by a second injection of 50 mg. of RC4 was given to the animals to obtain a high concentration. This was followed by a third injection of 2 mg. of



TEXT-FIGURE 4.—Survival of rats after the multiple-treatment schedules with RC4, Az., and TMCA, alone or in combination. For the doses used, *see* table 3.

TMCA and maintained by 20 mg. of RC4 from the fourth injection, followed by 2 mg. of TMCA alternately every 24 hours. During the period of treatment, a second large dose of drug was given to the animals when a few cells that were resistant to the drug started to grow again. The average survival of the animals was 25.7 days. However, at autopsy,

TABLE 3.—Multiple-treatment schedules with RC4, Az., and TMCA, alone and in combination

Experiment	Initial day of treatment*	Compound	Number of rats	Schedule of treatment	Daily dose (*mg./kg.*)	Number of injections†	Average survival time in days (*uncontrolled cases*)	Remarks
A	4–5	RC4	13	Every 24 hours	20	Min. 14 Max. 23	20. 5	All died
B	4–5	RC4	5	Every 24 hours	10	Min. 6 Max. 15	10. 8	All died
C	4–5	RC4	15	Every 12 hours	8 to 6‡	Min. 12 Max. 30	10. 2 (12 rats)	3 Cured
D	7–8	RC4	10	Every 24 hours	20	Min. 3 Max. 7	8. 3	All died
E	4–5	RC4 TMCA	20	Alternately every 24 hours	RC4, 20§ TMCA, 2	Min. RC4, 7 TMCA, 8 Max. RC4, 14 TMCA, 14	25. 7	All died‖
F	4–5	RC4 Az.	110	Every 24 hours	RC4, 20 to 10 Az., 5 to 2	Min. 8 Max. 12	16. 0 (101 rats)	9 Cured¶
G	4–5	RC4 Az.	13	Every 12 hours	RC4, 6** Az., 2	Max. 20	16. 4 (10 rats)	3 Cured
H	4–5	RC4 Az.	34	Every 12 hours	RC4, 6** Az, 2	Min. 24	18. 6 (9 rats)	25 Cured
I	7–8	RC4 Az.	9	Every 12 hours	RC4, 6** Az., 2	Min. 5 Max. 15	11. 0	All died
J	4–5	Az.	5	Every 24 hours	2	Min. 5 Max. 11	10. 4	All died

*Days after tumor inoculation (day 1 is day of inoculation).

†Min. = minimum number of injections. Max. = maximum number of injections.

‡The first dose was 20 to 10 mg., the second, 10 to 8 mg., the third, 8 to 6 mg., and the maintenance dose, 8 to 6 mg. per kg. of body weight, respectively.

§ The initial injection was TMCA, 4 mg., and the second, RC4, 50 mg., followed with the maintenance dose of TMCA, 2 mg., and RC4, 20 mg. per kg. of body weight, respectively.

‖ Five animals survived 26, 28, 29, 32, and 47 days after tumor inoculation. At autopsy no tumor was found.

¶ One rat died from pneumonia 30 days after tumor inoculation.

**Initial dose was RC4, 20 to 10 mg., and Az., 5 mg.; second and third dose, RC4, 10 to 8 mg. per kg. of body weight, respectively. RC4, 6 mg., and Az., 2 mg., were maintenance doses.

5 animals, which survived 26, 28, 29, 32, and 47 days after tumor-cell inoculation, showed no tumor.

There were scarcely any tumor cells in the ascitic fluid 3 to 4 days after injection. The tumor at the punctured site of the abdominal wall disappeared within 4 to 5 days and it became impossible to withdraw ascites on the 6th to 7th day. However, on the 10th to 12th day, tumor cells appeared in the ascites and there were signs of regrowth of the cells. When a second large dose of the drug was given to the animals, tumor cells again started to decrease. Most of the animals still showed a decrease in body weight and diarrhea on the 17th to 18th day. The tumor became resistant to treatment, and eventually the animals died from growth of the tumor or from weakness. The cytological changes did not differ much from those found with a single subcutaneous injection of RC4. Colchicine-like changes characteristic of those seen when TMCA was given were noted 24 hours after injection.

Combined repeated injection of RC4 and Az. every 24 hours (expt. F, table 3, and text-fig. 4).—RC4, 20 to 10 mg., and Az., 5 to 2 mg. per kg. of body weight, respectively, were both injected at the same time. Tumor cells decreased to half the number the day after injection. On the 2d day there were few tumor cells. By the 4th day, the tumor in the subcutaneous layer of the abdominal wall was reduced in size, and eventually disappeared. Only 1 to 2 tumor cells could be counted on the Thoma-type calculating chamber. About 1 week after injection there was only a slight decrease in body weight. However, it decreased rapidly around the 10th day. At this time the tumor cells started to increase again. Most of the rats died due to growth of the tumor or to weakness; 9 of 110 animals (8%) survived. Cytological findings were unlike the changes seen when a single injection of RC4 or RC4 plus TMCA was given. There were abnormal mitotic changes on the day after injection. On the 2d day, mitosis was scarcely recognized, while resting cells showed variant forms. Most of the tumor cells, which were relatively large, were transparent at the cellular margin, with vacuolization, outflow of cell protoplasm, and destruction of the nucleus. On the 4th day monocytes and leukocytes began to appear around the tumor cells. By the 9th and 10th day the animals could be roughly divided into 3 groups. In the first group there was a regrowth of the tumor, so that by the 12th and 13th day a condition of pure culture of tumor cells was again observed, with many mitotic cells. The animals died due to the growth of tumor, regardless of continued treatment. In the second group only scattered pyknotic cells were found. The tumor cells disappeared and the animals survived. In the third group there was a marked decrease in body weight and many had diarrhea, which made further treatment impossible. Parenteral injection of fluid did not prevent death from weakness or from growth of the tumor.

Repeated continued injection of RC4 and Az. every 12 hours (expt. H, table 3, and text-fig. 4).—The method of administering the drug was as follows: The initial doses of RC4, 20 to 10 mg., and of Az., 5 mg., were

used to give a large concentration. The second and third injections were carried out with RC4, 10 to 8 mg., and with 6 mg. of RC4 given as a maintenance dose from the fourth injection. This amount was the MED for subcutaneous use. Two mg. of Az. was used as a maintenance dose from the second injection, so that the animal would not become weakened even though prolonged continued injection was attempted. Every 12 hours 30 injections with these 2 substances were attempted simultaneously. Generally speaking, cytological changes were more noticeable than in the previous experiment. Mitosis decreased 12 to 48 hours after injection. Abnormal mitoses, *e.g.*, stickiness, scattering, coagulation, and fragmentation of chromosomes in metaphase, bridge formation, or delayed chromosomes in anaphase and telophase, were observed. Mitosis was scarcely noticed at 72 to 96 hours. On the other hand, resting cells were transparent at the cellular margin with vacuolization or pyknosis. Some degenerative changes were observed. On the 5th to 7th day there were fewer tumor cells. Many monocytes and leukocytes were found, but macrophages were not very active. All tumor cells disappeared after 27 to 28 injections. Twenty-five of 34 rats survived, for a survival rate of 73 percent. The cytological changes are illustrated in figures 1 to 4. No recurrence of tumor was noticed in the survivors up to 6 months after treatment. Two cured animals were killed on the 93d day, and each organ was carefully examined. No remarkable changes were found except slight glomerulitis. Nine animals died. In these, the cytological changes consisted of small tumor cells similar in size to monocytes, which started to increase about 10 days after treatment. On the 12th to 13th day, normal mitoses were observed and eventually the tumor cells appeared to be a pure culture. Despite continuing injection an intra-abdominal tumor appeared. A tumor became palpable at the subcutaneous puncture site of the abdominal wall, and the rat died from growth of the tumor.

The results with 13 rats (expt. G, table 3, and text-fig. 4) given 20 injections were similar to those with rats given 30 injections. However, after the injections were discontinued, most of the remaining tumor cells started to increase again and eventually the rats died from growth of the tumor. Only 3 animals continued to be free of tumor.

Treatment for animals in late stages (expt. D, table 3, and text-fig. 4, and expt. I, table 3, and text-fig. 4). When the rats were treated with RC4, 20 mg. per kg. of body weight every 24 hours, none survived and they died earlier. Combined therapy of RC4 and Az. every 12 hours also was not effective in prolonging life.

CLINICAL STUDY

Response of Patients With Advanced Cancer to Colchicine Derivatives

Materials and Methods

Four colchicine derivatives, trimethylcolchicinic acid methyl ether-D-tartrate (TMCA), thiocolchicine, colchiceinamide, and a condensation

product of colchiceine hydrazide with acetone (CPA) were administered intravenously. In some patients, especially when TMCA was used, injections were made into the pleural or abdominal cavity. TMCA was dissolved in water, while thiocolchicine, colchiceinamide, and CPA were dissolved in propylene glycol, which was added to the physiological saline solution.

The doses of TMCA, thiocolchicine, colchiceinamide, and CPA were 0.2 to 0.4 mg., 0.1 to 0.2 mg., 0.2 mg., and 0.2 to 0.3 mg. per kg. of body weight, respectively. Repeated doses were given daily or at intervals of 1 to 3 days. Of these compounds, TMCA and thiocolchicine were used chiefly.

Results

Fifteen patients, varying in age from 7 to 66 years and having a variety of disseminated neoplasms, were treated with colchicine derivatives. Of these, 3 patients received an additional combination chemotherapy with Nitromin. There were 9 patients with carcinoma and 6 with lymphoma, neuroblastoma, and Ewing's bone tumor. The data concerning the types of neoplastic disease and the results obtained are summarized in table 4. With the possible exception of 1 patient with carcinoma of the lung and 1 with lymphosarcoma, no therapeutic effects were noted despite the development of objective changes that were observed in 11 of the other 13 patients.

Case Report

Case 1, M.Y.—An operation was performed on a 56-year-old woman with carcinoma of the lung, which was diagnosed as anaplastic carcinoma. After middle-lobe lobectomy was carried out for primary cancer of the right lung that was confined to segment 4, many tumor cells were demonstrated in the effusion of the right pleural cavity on the 11th postoperative day. Mitoses were found in nearly 2 percent of tumor cells. With intrapleural injection of 20 mg. of TMCA, dissolved in 5 cc. of water, chromosomes in metaphase made the so-called ski pair and were in disorder, gradually accumulating with time. Ten mg. of thiocolchicine, dissolved in 2 cc. of propylene glycol, was injected into the pleural cavity 24 hours later. There was marked destruction and accumulation of cell division in metaphase in about 20 percent of tumor cells at the 33d hour. The type of destruction of metaphase which appeared during this time was chiefly scattered diplochromosomes, distorted star metaphase, ball metaphase, coagulation, and necrobiotic metaphase. Anaphase and telophase completely disappeared after the injection. Although the changes in resting cells were slight, giant cells appeared. After administration of thocolchicine, migration of leukocytes increased and phagocytosis of tumor cells was conspicuous. The tumor cells completely disappeared 48 hours later, as shown in table 5. It became impossible to obtain pleural effusion 15 days after the injection. When pleural injection was performed, the patient complained of severe chest pain, which was followed by a transient accumulation of exudate. Chest pain developed from the 3d hour after the injection and became increasingly severe but eventually disappeared when no pleural effusion could be obtained by means of puncture. The patient became asymptomatic and was discharged from the hospital about 2 months later. After about 1 year, she died with recurrence of the disease.

TABLE 4.—Response of patients with neoplastic

Case, age, and sex	Status prior to treatment: Duration disease (*months*)	Previous treatment	General condition	Site of major disease	Therapy: With colchicine derivatives: Type of colchicine derivatives	Therapy: With colchicine derivatives: Duration (*days*)
Carcinoma of lung						
1) M.Y., 56, female (anaplastic carcinoma)	3	Surgery, resection	Fair	Chest	TMCA, thiocolchicine	2
2) S.S., 45, male (anaplastic carcinoma)	3	—	Fair	Lungs, neck	TMCA	24
Carcinoma of stomach						
3) S.W., 45, male	2	—	Poor	Abdomen	TMCA, thiocolchicine, colchiceinamide	13
4) H.K., 43, female (carcinoma simplex)	4	Surgery, resection	Poor	Abdomen	TMCA	30
5) S.A., 50, female (carcinoma simplex)	36	Surgery, resection	Fair	Abdomen	Thiocolchicine, TMCA	18
6) T.U., 64, female (adenocarcinoma)	7	Surgery, resection	Poor	Abdomen	TMCA, colchiceinamide	22
Carcinoma of small intestine						
7) S.W., 61, male (small cell carcinoma)	2	X ray, Nitromin	Poor	Abdomen	TMCA	4
Carcinoma of thyroid						
8) H.M., 63, male (papillary carcinoma)	18	Surgery	Fair	Neck, chest	TMCA, thiocolchicine, colchiceinamide, colchiceine hydrazide with acetone, colchicine	176
Carcinoma of breast						
9) M.M., 38, female (scirrhous carcinoma)	11	—	Fair	Nodes	TMCA	47

disease treated with colchicine derivatives

Therapy—Con.					
With colchicine derivatives—Con.	Combined with Nitromin				
Total (*mg.*)	Duration (*days*)	Total (*mg.*)	Response	Subsequent course	Follow up after start of therapy with colchicine derivative
20 10			Disappearance of pleural effusion	Colchicine derivatives discontinued after disappearance of pleural effusion	Died, 12 months
220	24	900	Slight regression of tumor mass on neck	Therapy discontinued without remarkable improvement; no follow up	1 Month
50 5 30			Disappearance of abdominal fluid	Died during therapy	Died, 1 month
140			Disappearance of tumor cells in abdominal fluid	Died during therapy	Died, 2 months
60 30			Regression of abdominal mass; operated again	Colchicine derivatives discontinued because of hematopoietic depression; no follow up	1 Month
40 30	15	300	Slight repression of abdominal mass; subjective improvement	Treatment discontinued because of hematopoietic depression; no follow up	1 Month
60			Total necrosis of intestinal tumor mass and metastases in abdominal wall	Died during therapy	Died, 4 days
120 44 42			Temporary decrease of pleural effusion	Died during therapy	Died, 6 months
60 15					
220	43	700	Regression of lymph node	Treatment discontinued because of hematopoietic depression; no follow up	1½ Months

TABLE 4.—Response of patients with neoplastic

Case, age, and sex	Status prior to treatment				Therapy	
	Duration disease (*months*)	Previous treatment	General condition	Site of major disease	With colchicine derivatives	
					Type of colchicine derivatives	Duration (*days*)
Reticulum-cell sarcoma						
10) J.B., 66, male	10	X ray	Poor	Generalized	TMCA	4
11) N.A., 7, male	8	X ray	Fair	Generalized	TMCA, thiocolchicine, colchiceinamide, colchiceine hydrazide with acetone	49
12) C.K., 57, male	2	—	Fair	Generalized	TMCA	55
Lymphosarcoma						
13) S.A., 59, female	6	Nitromin	Fair	Nodes	TMCA	27
Neuroblastoma of face						
14) I.A., 9, male	2	X ray	Fair	Face	TMCA, thiocolchicine	8
Ewing's bone tumor						
15) Y.S., 18, male	9	—	Poor	Femur	TMCA	9

Toxic Effect of Colchicine Derivatives

TMCA, dissolved in water, and the other 3 colchicine derivatives, dissolved in propylene glycol and added to the saline solution, could be administered intravenously. There was no immediate or delayed reaction locally due to the injection. When the dose was reduced below a critical level, undesirable reactions were infrequent. However, when TMCA or thiocolchicine was injected intrapleurally or intra-abdominally, severe chest pain or abdominal pain developed. Of 3 patients treated intrapleurally or intra-abdominally, 2 (case 7 and 10) died in a state of shock with severe abdominal pain within 12 hours. Therefore it may be dangerous to inject these substances intra-abdominally.

The most significant toxic manifestation from the colchicine derivatives was bone marrow depression. It became evident that the tumor-inhibiting effect of the colchicine derivatives could not be separated from its depressing effect on bone marrow.

disease treated with colchicine derivatives—Continued

Therapy—Con.					
With colchicine derivatives—Con.	Combined with Nitromin				
Total (*mg.*)	Duration (*days*)	Total (*mg.*)	Response	Subsequent course	Follow up after start of therapy with colchicine derivative
40			Appearance of many ball metaphases with active phagocytosis in abdominal fluid	Died during therapy	Died, 4 days
93 45 10 10			Temporary regression of tumor mass	Colchicine derivatives discontinued because of appearance of resistant tumor mass	Died, 4 months
220			Temporary regression of tumor mass	Colchicine derivatives discontinued because of appearance of resistant tumor mass; no follow up	2 Months
90			Regression of tumor mass	Quite well without any treatment	24 Months
40 20			None	No follow up	½ Month
90			None	No follow up	½ Month

Response of Patients With Advanced Cancer to RC4 Combined With TMCA

Materials and Methods

At the outset of the clinical trials RC4, which was available as a dried powder, was dissolved in 5 percent glucose in water and given intravenously. The drug was prepared in a concentration of 5 to 20 mg. per ml. and administered without any untoward effect. The dose of RC4 was 1 to 4 mg. per kg. of body weight and repeated doses were given daily or every other day. In 3 patients, only RC4 was administered by local injection into tumor nodules.

TMCA was dissolved in water and prepared in a concentration of 10 mg. per ml. and given intravenously, as previously described. The dose of TMCA was 0.2 mg. per kg. of body weight. When TMCA was used in

TABLE 5.—Carcinomatous pleural effusion in 56-year-old female with lung cancer

Treatment	Counted numbers of tumor cells	Numbers of normal pro- and metaphase	Numbers of abnormal metaphase	Numbers of normal ana- and telophase
Before injection	1000	4	0	15
Injection	Trimethylcolchicinic acid methyl ether-D-tartrate, 20 mg. intrapleural			
After:				
3 hours	500	1	10	0
6 hours	500	0	46	0
9 hours	250	0	27	0
24 hours	Thiocolchicine, 10 mg. intrapleural injection			
27 hours	40	0	4	0
30 hours	150	0	23	0
33 hours	88	0	20	0
48 hours	0	0	0	0
6 days	0	0	0	0
7 days	0	0	0	0
10 days	0	0	0	0
15 days	Pleural effusion is not obtained			

conjunction with RC4, the former was administered at intervals of 1 to 3 days.

Results

Fourteen patients with inoperable or far-advanced cancer were treated. There were 10 patients with carcinoma and 4 with lymphoma. The data concerning the types of neoplastic disease and the results obtained are summarized in table 6. In only 1 patient, who had postoperative carcinomatous pleurisy, was there any clinical remission, though some objective changes were observed in 5 of the remaining 10 patients after administration of the combination chemotherapy with RC4 and TMCA.

Case Report

Case 16, C.M.—A 47-year-old man with adenocarcinoma of the esophagus had a radical operation. However, many tumor cells were found in the effusion of the left pleural cavity on the 15th postoperative day. During the following 10 days, he received intrapleurally a total of 1100 mg. of RC4 and 20 mg. of TMCA. It became impossible to obtain pleural effusion 10 days after the injection. He left the hospital and was quite well 8 months after the combination chemotherapy of RC4 and TMCA.

Undesirable Effects

There was no immediate or delayed reaction locally due to intravenous injection of RC4 and TMCA combined or RC4 alone. Also there was no immediate systemic reaction of nausea, vomiting, or anorexia. However, treatment was not continued in a majority of the patients because toxic manifestation in bone marrow developed.

Clinical Studies of Combination Chemotherapy With RC4 and Az. in the Treatment of Inoperable or Advanced Cancer

Method of Treatment

RC4 and Az. were administered intravenously. RC4 was dissolved in 1 ml. of propylene glycol and prepared in a concentration of 25 mg.; Az. was dissolved in 5 ml. of water and prepared in a concentration of 5 mg. These 2 substances, diluted together in 100 ml. of 5 percent glucose in water or in physiological saline solution, could be administered without any adverse effect. Pain along the vein occurs when undiluted Az. is injected.

The schedules of dosages and time intervals of the administration of the compounds were changed several times in an attempt to diminish, as much as possible, the deleterious effect of their long-term continued injection. In the early part of this investigation, from February, 1957, to May, 1957, 1 mg. of RC4 and 0.5 to 0.2 mg. of Az. per kg. of body weight, respectively, were given intravenously each day. From June, 1957, to August, 1957, 1 to 0.5 mg. of RC4 and 0.2 mg. of Az. per kg., of body weight, respectively, were administered twice a week. After the intravenous dosage schedule was established, treatment of patients who had inoperable or advanced cancer was begun in September, 1957, with 0.5 mg. of RC4 and 0.1 mg. of Az. per kg. of body weight, respectively, twice a week.

Treatment and Results

Thirty-one patients with inoperable or advanced cancer, from 27 to 73 years of age, were treated with a combination of RC4 and Az. There were 27 patients with carcinoma and 4 with lymphoma and malignant melanoma. Six patients, case 47 to 52, were treated by Dr. Akaishi in another clinic. All cancer patients undergoing surgery for potentially curable lesions were excluded in this report.

Not all the patients who were started on the combination therapy received an adequate trial of therapy. The main reasons were that the amount of the daily administered drugs produced definite signs of toxicity or the patients did not desire to remain in the hospital for a long period of treatment. Table 7 summarizes the data on the types of inoperable or advanced cancer and the results obtained. Of these 31 patients, objective or subjective improvement was observed in 24 of 27 patients with carcinoma, whereas therapeutic effects were scarcely noted in 4 patients with lymphoma and malignant melanoma.

Case Reports

Case 30, S.S.—A 47-year-old woman had a radical mastectomy, in July, 1956, for carcinoma simplex of the right breast with regional-node metastasis. At the end of 10 months she developed subcutaneous recurrent tumor nodules over the anterior chest wall on the right side and right pleural effusion. Aspirated fluid from the pleural cavity showed tumor cells. There was also a palpable, right supraclavicular node. She was referred to us for the combination chemotherapy in June, 1957. During the first 8 weeks she received a total of 950 mg. of RC4 and 10 mg. of Az.,

TABLE 6.—Response of patients with neoplastic disease treated with combination therapy of RC4 and TMCA

Case, age, and sex	Status prior to treatment: Duration disease (*months*)	Previous treatment	General condition	Site of major disease	Therapy: With RC4 and TMCA and RC4 alone: Number doses	Total (*mg.*)	Duration (*days*)	Response	Subsequent course	Follow up after start of combination therapy with RC4 and TMCA
Carcinoma of esophagus										
16) C.M., 47, male (adenocarcinoma)	6	Surgery, resection	Poor	Chest	RC4, 9 TMCA, 2	1100 20	10	Disappearance of pleural effusion	Well, 10 months	10 Months
17) G.T., 71, male (carcinoma simplex)	5	—	Poor	Abdomen, chest	RC4, 3 TMCA, 2	300 20	6	None	Died during therapy	Died, 8 days
Carcinoma of stomach										
18) A.A., 24, female (adenocarcinoma)	6	Surgery, resection	Fair	Abdomen	RC4, 14 TMCA, 7	1400 70	21	None	Combination therapy with RC4 and TMCA discontinued*; no follow up.	⅔ Month
19) C.E., 39, male (adenocarcinoma)	3	Surgery, resection	Poor	Abdomen	RC4, 14 TMCA, 5	2100 50	14	None	Combination therapy with RC4 and TMCA discontinued*; died during therapy	Died, 1 month
Carcinoma of biliary tract										
20) T.K., 72, male (adenocarcinoma)	1	—	Poor	Abdomen	R4C, 12 TMCA, 3	1200 30	13	None	Died during therapy	Died, 14 days
21) C.T., 45, male (adenocarcinoma)	16	Surgery, resection	Poor	Abdomen	RC4 alone, 10	900	30	Temporary disappearance of abdominal pain	Died during therapy	Died, 1 month

Carcinoma of breast										
22) S.O., 48, male (adenocarcinoma)	15	—	Fair	Skin, nodes.	RC4, 15 TMCA, 6	1900 60	18	None	Combination therapy with RC4 and TMCA discontinued*; no follow up	2 Months
Bronchial carcinoma										
23) S.Y., 72, male (squamous-cell carcinoma)	18	—	Fair	Neck	RC4, 18 TMCA, 5	1800 50	15	Diffuse tumor slough	Combination therapy with RC4 and TMCA discontinued*; progressive decline; died during therapy	Died, 1 month
24) T.M., 58, male (squamous-cell carcinoma)	1	—	Poor	Neck, nodes	RC4 alone, 19	950	21	None	Died during therapy	Died, ⅓ month
Carcinoma of neck (unknown primary site)										
25) C.A., 44, female (squamous-cell carcinoma)	2	Nitromin, sarcomycin	Poor	Neck, lung, nodes	RC4, 16 TMCA, 8	1500 80	47	Temporary regression of tumor mass	Combination therapy with RC4 and TMCA discontinued*; progressive decline	Died, 3 months
Reticulum-cell sarcoma										
26) K.A., 63, female	1	—	Poor	Neck	RC4, 26 TMCA, 8	2600 80	30	Shrinkage of lymph nodes	Combination therapy with RC4 and TMCA discontinued*; died during therapy	Died, 1 month
27) U.K., 31, male	5	—	Fair	Neck	RC4, 20 TMCA, 5	1000 50	38	Shrinkage of lymph nodes	No follow up	2 Months
28) Y.O., 54, female	3	—	Fair	Nodes	RC4 alone, 29	1450	29	Temporary shrinkage of lymph nodes	No follow up	1 Month
29) K.K., 42, female	1	—	Fair	Neck	RC4, 22 TMCA, 6	1100 60	40	Temporary shrinkage of lymph nodes	No follow up	2 Months

*Combination therapy with RC4 and TMCA was discontinued because of hematopoietic depression.

TABLE 7.—Response of patients with neoplastic

Case, age, and sex	Status prior to treatment				Site of major disease	Combination with RC	
	Duration disease (*months*)	Last previous therapy	Initial therapy	General condition		Number doses	
Carcinoma of breast							
30) S.S., 47, female (carcinoma simplex)	12	—	Radical mastectomy	Fair	Chest, nodes	RC4 Az.	30
31) S.T., 62, female (carcinoma simplex)	6	—	Simple mastectomy	Fair	Nodes	RC4 Az.	17
32) S.M., 41, female (adenocarcinoma)	9	—	Supraradical mastectomy	Fair	Chest, neck, nodes	RC4 Az.	14
33) S.O., 45, female (carcinoma simplex)	11	Radical mastectomy; massive roentgen therapy	—	Poor	Chest, nodes	RC4 Az.	5
Carcinoma of lung							
34) K.Y., 73, male (carcinoma simplex)	4	—	—	Fair	Lung	RC4 Az.	38
35) M.A., 62, male (carcinoma simplex)	8	—	—	Poor	Lungs, nodes	RC4 Az.	22
36) E.G., 59, male (oat-cell cancer)	9	—	—	Poor	Chest, bones	RC4 Az.	9
37) K.T., 61, male (carcinoma simplex)	12	Nitromin	—	Fair	Chest	RC4 Az.	29
38) N.O., 64, male (adenocarcinoma)	12	—	—	Poor	Lung, nodes, skin	RC4 Az.	9
Carcinoma of stomach							
39) E.T., 42, male (adenocarcinoma)	4	—	Surgery, exploration	Fair	Abdomen	RC4 Az.	82

disease treated with RC4 and Az.

tion therapy 4 and Az.				
Total (*mg.*)	Duration (*days*)	Response	Subsequent course	Follow up after start of combination therapy with RC4 and Az.
1225 290	94	Disappearance of lymph nodes; disappearance and regression of skin nodules; disappearance of pleural effusion; well, 9 months	Combination therapy with RC4 and Az. discontinued because of slight impairment of liver function	Died, 11 months
575 170	57	Regression of lymph nodes; inadequate and incorrect therapy; well, 11 months	Combination therapy with RC4 and Az. discontinued because of impairment of liver function and hematopoietic depression; testerone, X-rays; fair response	Died, 12 months
350 70	47	Well, 2 months; inadequate therapy	At follow-up study, progressive decline after suspension of combination chemotherapy	Died, 7 months
125 25	15	None; inadequate therapy	Oophorectomy; temporary response during only 10 days; no response with androgen	1 Month
2600 380	78	Temporary regression of tumor mass; inadequate and incorrect therapy	Combination therapy with RC4 and Az. discontinued because of hematopoietic depression and jaundice	Died, 3 months
1100 220	74	Regression of tumor mass; inadequate trial	No follow up	2½ Months
450 990	9	Spontaneous regression of tumor mass; inadequate therapy	Combination therapy with RC4 and Az. discontinued because of remittent high fever; progressive decline	Died, 3 months
725 195	99	Temporary decrease of pleural effusion	Combination therapy with RC4 and Az. discontinued because of no improvement; progressive decline	Died, 4 months
225 45	32	Subjective improvement with amelioration of chest pain	Combination therapy with RC4 and Az. discontinued because of no objective improvement	1½ Months
1925 405	274	Regression of abdominal mass; operation was performed; well, 12 months	Combination therapy with RC4 and Az. discontinued because of financial condition of the patient	Died, 14 months

TABLE 7.—Response of patients with neoplastic

Case, age, and sex	Status prior to treatment					Combina- with RC	
	Duration disease (*months*)	Last previous therapy	Initial therapy	General condition	Site of major disease	Number doses	
Carcinoma of stomach—Con.							
40) N.S., 27, male (carcinoma simplex)	7	—	Surgery, resection	Fair	Abdomen	RC4 Az.	14
41) R.O., 45, male (carcinoma simplex)	14	Surgery, resection	Surgery, total gastrectomy	Fair	Abdomen	RC4 Az.	13
42) K.T., 47, male (carcinoma simplex)	6	—	Surgery, resection	Poor	Abdomen	RC4 Az.	14
43) M.I., 52, male (carcinoma simplex)	6	—	—	Poor	Abdomen	RC4 Az.	7
44) K.S., 51, male (adenocarcinoma)	15	Surgery, resection	Surgery, exploration	Poor	Abdomen	RC4 Az.	22
45) K.S., 57, male (adenocarcinoma)	5	—	Surgery, resection	Poor	Abdomen	RC4 Az.	16
46) K.K., 52, male (colloid carcinoma)	24	Surgery, resection	—	Poor	Abdomen	RC4 Az.	39
47) T.F., 37, female (carcinoma simplex)	10	—	Surgery, total gastrectomy	Poor	Abdomen	RC4 Az.	23
48) I.K., 53, male (carcinoma simplex)	2	—	Surgery, total gastrectomy	Fair	Abdomen	RC4 Az.	23
49) Y.A., 68, male (carcinoma simplex)	6	—	Surgery, exploration	Poor	Abdomen	RC4 Az.	18
50) T.M., 35, female (carcinoma simplex)	3	—	Surgery, total gastrectomy	Poor	Abdomen	RC4 Az.	43
51) M.A., 47, female (carcinoma simplex)	1	—	Surgery, resection	Fair	Abdomen	RC4 Az.	14
52) C.D., 51, female (adenocarcinoma)	12	Surgery, resection	—	Poor	Abdomen	RC4 Az.	15

disease treated with RC4 and Az.—Continued

tion therapy 4 and Az.				
Total (*mg.*)	Duration (*days*)	Response	Subsequent course	Follow up after start of combination therapy with RC4 and Az.
375 125	53	Marked increase of body weight; inadequate therapy; well, 3 months	At follow-up study, progressive decline after suspension of combination chemotherapy	Died, 6 months
325 65	43	Well, 2 months; inadequate therapy	No follow up	2 Months
350 70	57	Well, 2 months; inadequate therapy	No follow up	2 Months
350 70	7	Perforation of gastric carcinoma	Died during therapy	Died, ⅔ month
750 205	64	None	Progressive decline	Died, 2 months
400 80	56	Suppression of ascites accumulation; well, 2½ months	Combination therapy with RC4 and Az. discontinued because of impairment of liver function; no follow up	2½ Months
975 195	134	No suppression of ascites accumulation	Died during therapy	Died, 4½ months
575 115	132	Well, 5 months; inadequate therapy	Operation was done over again in 5 months, after initial combination therapy with RC4 and Az. because of intestinal ileus; snow-like metastases, 2 × 2 mm. in size, over all the small bowel and the mesentery, without ascites	Died, 8 months
575 115	88	Well 9 months; inadequate therapy		Living, 9 months
450 90	66	Well, 2 months; inadequate therapy	No follow up	2 Months
1075 215	150	Suppression of ascites accumulation; well, 9 months	Progressive decline 10 months after initial combination therapy with RC4 and Az.	Died, 12 months
350 70	58	Well, 2 months; inadequate therapy	Progressive decline	Died, 3½ months
375 75	60	No suppression of ascites accumulation	Progressive decline	3 Months

TABLE 7.—Response of patients with neoplastic

Case, age, and sex	Status prior to treatment: Duration disease (*months*)	Last previous therapy	Initial therapy	General condition	Site of major disease	Combina- with RG: Number doses	
Carcinoma of esophagus							
53) J.T., 43, male	7	—	—	Poor	Chest	RC4 Az.	31
54) S.S., 60, male (cancroid)	6	Surgery, resection	—	Poor	Chest	RC4 Az.	31
55) Y.W., 70, male (squamous-cell carcinoma)	20	Surgery, resection	—	Fair	Chest, nodes	RC4 Az.	30
Carcinoma of pancreas							
56) F.M., 32, female (adenocarcinoma)	3	—	Surgery, exploration	Fair	Abdomen	RC4 Az.	19
Carcinoma of parotid							
57) S.W., 56, male	3	—	—	Fair	Parotid	RC4 Az.	4
Malignant melanoma							
58) Y.I., 67, male	5	—	—	Fair	Nodes, abdomen, lungs	RC4 Az.	7
Hodgkin's disease							
59) T.T., 30, male	7	—	—	Poor	Nodes	RC4 Az.	21
Reticulum-cell sarcoma							
60) S.O., 54, male	6	—	—	Poor	Generalized	RC4 Az.	14
61) M.T., 47, female	2	—	—	Fair	Neck	RC4 Az.	6

disease treated with RC4 and Az.—Continued

tion therapy 4 and Az.				
Total (*mg.*)	Duration (*days*)	Response	Subsequent course	Follow up after start of combination therapy with RC4 and Az.
950 225	92	Can swallow solid food; perforation of esophageal carcinoma	Died during therapy	Died, 3 months
775 155	110	Perforation of esophageal carcinoma	Died during therapy	Died, 3½ months
750 150	105	Well, 3½ months	Now under combination therapy with RC4 and Az.	Living, 3½ months
475 95	64	Disappearance of abdominal pain; well, 2 months	Combination therapy with RC4 and Az. discontinued because of impairment of liver function; no follow up	3 Months
200 40	8	Regression of tumor mass; inadequate therapy	X ray; fair response	7 Months
350 100	7	None; inadequate therapy	No follow up	½ Month
1050 255	21	Temporary regression of lymph node	Combination therapy with RC4 and Az. discontinued because of absence of improvement; cortisone, colchicine derivatives, temporary response; progressive decline	Died, 6 months
700 140	44	None	Died during therapy	Died, 2 months
150 60	16	None	X ray; fair response	5 Months

respectively, and in the following 6 weeks, 11 single doses of 25 mg. of RC4 and 10 mg. of Az., respectively. At the end of this period there was a definite decrease of pleural effusion and disappearance of the subcutaneous tumor nodules. However, 12 days after the treatment was discontinued a solitary small nodule, 0.5×0.3 cm., was palpable on the right anterior chest wall and a specimen showed that a few tumor cells still remained. She left the hospital, and a follow up revealed that she died 11 months after the initial combination chemotherapy.

Case 35, M.A.—A 62-year-old man had a large, fixed lump in the right supraclavicular region and matted nodes in the left supraclavicular region, for 9 months prior to admission. He also complained of pain in the right neck and the right breast. On roentgen-ray examination, carcinoma of the right lung, which was confined to segments 4, 5, and 6, was found. There were many tumor cells in his sputum. In May, 1957, he was referred to us for combination chemotherapy. After receiving intermittently a total of 650 mg. of RC4 and 130 mg. of Az. in 38 days, there was a definite decrease in the size of the right supraclavicular mass and it became movable. A marked decrease in intensity and frequency of cough, expectoration of sputum, and pain in the right neck region was observed. The right supraclavicular mass was removed 44 days after the initial treatment, and the pain in the right neck region completely subsided. The microscopic picture, diagnosed as carcinoma simplex, showed that remarkable changes were not found during a short period of the treatment in spite of the macroscopic reduction of the tumor size. However, a chest roentgenogram revealed a marked improvement compared with the one made prior to therapy. Because of his financial situation, the patient left the hospital without adequate combination chemotherapy. He had received a total of 1100 mg. of RC4 and 220 mg. of Az. in 74 days, without any signs of progressive liver impairment or systemic toxicity which could be attributed to the combination chemotherapy. A follow-up study was not obtained.

Case 39, E.T.—In June, 1957, a 42-year-old man had an exploratory laparotomy, in another hospital, and an inoperable gastric adenocarcinoma was found. He was admitted to our clinic and 17 days after the previous operation a laparotomy was done again. At that time a tumor mass, 8×8 cm. in diameter, was found in the pyloric region and there was firm adhesion of neoplastic tissue of the stomach to the head of the pancreas. Moreover, the superior mesenteric artery was surrounded by neoplastic tissue. Radical operation was abandoned and the patient was referred for combination chemotherapy. In the following 35 days, he received intermittently a total of 750 mg. of RC4 and 150 mg. of Az. Forty-three days after the second operation the third laparotomy was attempted. Two tumor masses were found in the pyloric region, of which one measured 6×4 cm. in diameter and another 4×3 cm. There was scarcely any adhesion of the head of the pancreas with the stomach or neoplastic invasion around the superior mesenteric artery. A tumor, the size of a pigeon's egg, was found on the lower edge of the posterior surface of the pancreas. Subtotal gastrectomy was performed, without extirpating the tumor behind the pancreas. However, microscopic examination of biopsies from the lesion did not show striking changes, though the macroscopic reduction of the tumor size was obtained. In the following 9 months he received intermittently a total of 1175 mg. of RC4 and 255 mg. of Az. He died 14 months after the initial combination chemotherapy.

Case 40, N.S.—In July, 1957, a 27-year-old man had a total gastrectomy for carcinoma simplex of the cardia. At that time neoplastic invasion, involving the retroperitoneal regions, omentum, diaphragm, left liver, and hilum of the spleen, was found. In the following 53 days, he received intermittently a total of 375 mg. of RC4 and 125 mg. of Az. in 14 single intravenous injections of 50 to 25 mg. of RC4 and 10 to 5 mg. of Az. He was in a very good condition when last seen in November, 1957, and had gained 6 kg. He stopped the combination chemotherapy, though he was advised to continue a long-term treatment. Our follow-up study revealed that he died in January, 1958, after a progressive deterioration.

Toxic Effects of the Combination Chemotherapy

RC4 and Az., diluted in 100 ml. of 5 percent glucose in water or physiological saline solution, were administered intravenously without any immediate or delayed adverse effect, including venous thrombosis. There was also no immediate untoward reaction of nausea, vomiting, or anorexia.

An appreciable decrease in the leukocyte count did not occur in 21 of 24 patients who received repeated injections over 44 days. In two instances (case 31 and 34) in the early part of this investigation, a leukopenia of sufficient degree did develop to interdict the further administration of the combination chemotherapy after daily repeated injections had been given. After the intravenous dosage schedule was established, only 1 patient (case 47) showed a moderate leukopenia when therapy was carried out twice a week, which necessitated frequently reinstituting the therapy after the blood returned to normal.

Anemia was observed in a few cases after an interval of 1 to 2 months. However, the blood could be easily restored to normal by several transfusions, 200 cc. per day.

In the earlier part of this investigation, 2 patients (case 31 and 34) showed jaundice or progressive impairment of liver function accompanied by an increase in bromsulfalein retention after receiving daily or intermittent administration of the combination chemotherapy. To prevent possible liver disturbance, glucuronic acid, 2 to 4 mg. per kg., and vitamin K, 2 mg. per kg., were both administered daily. After the intravenous dosage schedule was established, only 2 patients (case 45 and 56) among those who had a long-term combined injection twice a week showed jaundice with bromsulfalein retention. Patient 45 showed increase of BSP, 35 percent at 30 minutes, 1½ months after beginning the chemotherapy. Patient 56 also showed the same tendency—BSP 30 percent at 30 minutes after 2 months of treatment. However, both of them had enlarged lymph nodes of cancer origin around the bile duct and portal-vessel system when operated upon. It was not clear whether or not the BSP retention was due to the enlargement of these lymph nodes obstructing the biliary system. The 2 patients, just mentioned, were the only ones whose therapy had to be suspended due to disturbance of liver function.

DISCUSSION

The substances that have been effective in human cancer have caused changes in Yoshida sarcoma, with the exception of steroids and the so-called antimetabolites. According to our laboratory and clinical studies, a long-term combined therapy of RC4 and Az. seemed to produce the best results.

It can be postulated that human cancer may be controlled effectively with chemotherapy if such therapy is instituted early, but it is not always possible to treat patients in the incipient period. It is naturally desirable

to initiate chemotherapy after potentially curable lesions are removed by operation, but it will take 5 or 10 years to obtain follow-up results. Therefore, when patients with far-advanced cancer are treated by chemotherapy some data may be procured to judge the effectiveness of cancer chemotherapy. Treatment may not be successful if there are numerous cancer cells, as was shown in the experiments with rats that were in a late stage of the disease. Therefore, it may be desirable to begin chemotherapy after extirpating the main tumors.

At the present time the most important problem in cancer chemotherapy is whether the tumor-inhibiting effect of chemicals can be separated from their depressing effect on bone marrow, digestive-tract disturbances, and systemic toxicity with or without liver damage, etc. The main reason that the so-called anticancer drugs are unpopular with many clinicians may be due to the toxic effect of these chemicals even if objective and subjective improvement were acquired. Consequently, untoward effects should be eliminated as far as possible without destroying the tumor-inhibiting effect of chemicals.

A great majority of patients who were benefited by the combination chemotherapy of RC4 and Az. did not develop toxic changes when the treatment schedule was established. Moreover, in patients with inoperable or disseminated cancer, the production of objective and subjective changes in those with carcinoma of the breast, lung, and digestive tract, after treatment with the combination chemotherapy of RC4 and Az., yields preliminary evidence for the carcinostatic properties of these compounds.

In view of the animal experiments and the clinical research, it is apparent that for the combined cancer chemotherapy to be effective a long-term treatment schedule is required, as is true for the present tuberculosis chemotherapy. Additional emphasis should be placed on this important problem, and it is necessary to educate both doctors and patients because they tend to stop the combination chemotherapy prematurely, though the long-term treatment is more effective. However, even though 30 repeated injections are required in the animal experiment, it is difficult to say definitely how many repeated injections are required in each human cancer. At present it is not clear whether the treatment schedule of injections carried out twice a week is right for each human cancer, though the time interval of injection was found to be important in the animal experiments.

Considering the unsatisfactory results of our therapy, the efficiency of RC4, one of the alkylating agents which is a major factor in combined chemotherapy, may not yet have reached therapy efficiency in clinical cancer. The MTD against the MED of RC4 by subcutaneous injection on Yoshida sarcoma is only 13. Az., which is considered to be an antimetabolite, may be working only as a supplementary substance to depress the occurrence of resistant tumor cells.

In order to have more powerful compounds, alkylating agents and amino acids with an unsaturated 7-membered ring structure have been synthesized, and are now under animal investigation.

With our present combined therapy, despite macroscopic decrease in the size of the tumor, there was no total fibrosis or necrosis by histologic study, with the possible exception of 1 patient (case 7). Microscopically, a number of patients have shown fibrosis, necrosis, or cell degeneration to a higher or lower degree. However, such changes can be observed in some patients with malignant tumors under no treatment, which makes it hard to judge the cause of such changes. Also, as indicated in 3 patients, the application of cancer chemotherapy is dangerous when there is carcinoma of the digestive tract because the combination chemotherapy might induce perforation.

According to our experience, the combination chemotherapy as an adjunct to surgery may have considerable significance in the treatment of patients with advanced cancer.

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PLATES

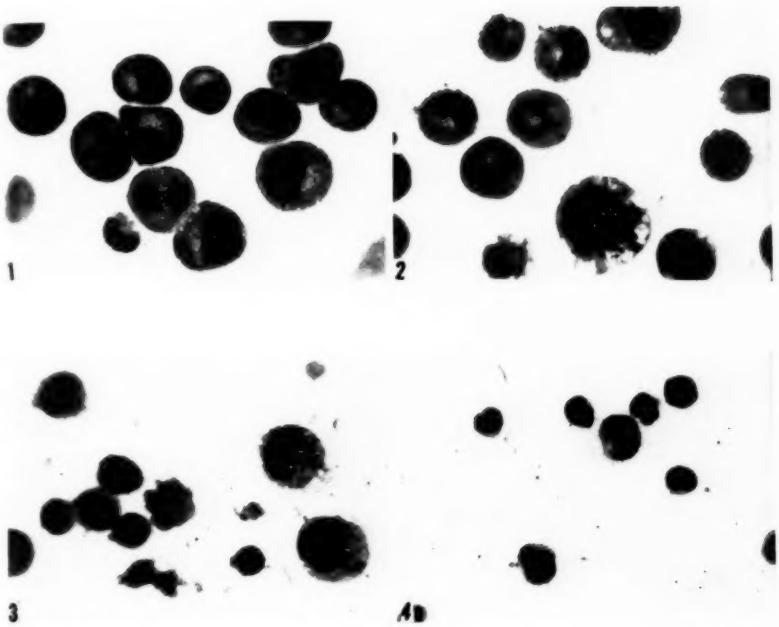
PLATE 6

Cytomorphological changes in Yoshida ascites tumor cells, after combination chemotherapy with Az. and RC4. Giemsa. × 950

FIGURE 1.—Tumor cells in pure culture, 72 hours after tumor-cell inoculation.

FIGURES 2 AND 3.—Cellular degeneration, especially vacuolar formation in cytoplasm in resting cells, 72 hours and 10 injections after combination chemotherapy.

FIGURE 4.—No tumor cells are seen after 30 injections with combination chemotherapy.



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Transplantation of Homologous and Heterologous Lymphoid Cells in X-Irradiated and Nonirradiated Mice[1]

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SUMMARY

The injection of a large number of homologous lymphoid cells kills X-irradiated mice protected with isologous or homologous bone marrow, within 1 to 2 weeks. The injection of a smaller number of homologous lymphoid cells kills the mice after a longer interval. Parental-strain lymphoid cells have a detrimental effect in irradiated F_1 hybrids, but F_1 hybrid lymph-node cells produce no ill effect in parental strain mice. Rat lymph-node cells only have a lethal effect in irradiated mice protected with heterologous bone marrow when the cells are injected at 24 hours after irradiation. On the basis of the number of cells required to produce a certain percentage of mortality, homologous lymph-node cells are somewhat more effective than homologous spleen cells, but much more effective than homologous thymus cells. Mice that died 10 to 20 days or later after injection of homologous or heterologous lymphoid cells had symptoms and lesions which resembled those seen in mice that died from the secondary disease after foreign bone marrow transplantation. A pathological syndrome resembling the secondary disease has been evoked in a certain number of nonirradiated F_1 hybrid mice by the injection of a large number of lymphoid cells from parental strain mice that had been sensitized against isoantigens of the other parental strain. These observations support the concept of a graft-antihost reaction that is involved in the development of secondary disease.—J. Nat. Cancer Inst. 23: 53–73, 1959.

SINCE 1955 different groups of investigators (*1–5*) have shown that a transplantation of homologous and heterologous bone marrow can be achieved in mice after their irradiation with a lethal X-ray dose. Since the injected blood-forming cells proliferate and repopulate the hematopoietic organs (*6, 7*), death from bone marrow failure is prevented. The production of erythrocytes (*1, 2, 5*), granulocytes (*3, 5*), thrombocytes (*8*), and lymphoid cells (*9, 10*) of donor origin has been demonstrated in the survivors. With regard to the proliferation of donor-type lymphoid

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cells, the available data are only semiquantitative, but undoubtedly the lymphoid tissues are repopulated by the grafted cells. Congdon and Urso (*11*) made a histological study of the hematopoietic tissues of lethally irradiated mice grafted with homologous bone marrow. They observed that the recovery of the lymphatic tissues occurred much later than the restoration of the other hematopoietic organs. This study has been fully confirmed by our observations (*12*).

The secondary disease (foreign bone marrow disease) and delayed death that occur in irradiated mice after treatment with homologous or heterologous bone marrow have been attributed by some investigators to a graft-antihost reaction (*13–16*). Others have postulated a host-antigraft reaction as the cause of the secondary disease (*17*).

With regard to the first hypothesis, it seemed of interest to collect additional information on the consequences of foreign lymphoid cell proliferation to the host. Since a slow restoration of the lymphatic tissue is apparently the result of the proliferation of a few blood-forming stem cells from the transplanted bone marrow, a more rapid lymphoid cell proliferation might be expected when lymphoid cells and bone marrow cells are grafted. Therefore, a varying number of lymph-node, spleen, or thymus cells were injected, in addition to the usual amount of bone marrow, into lethally irradiated homologous or heterologous hosts.

In the course of our investigation, the injection of homologous spleen cells into irradiated mice was reported to be detrimental (*18–20*), and the death of irradiated mice that received homologous lymph-node cells in addition to homologous bone marrow was noted by others (*21*). The data presented here confirm and extend these observations.

MATERIALS AND METHODS

Animals.—Mice were bred in the laboratory by brother × sister mating in the highly inbred CBA and C57BL strains.[5] The F_1 hybrid was derived from CBA males and C57BL females. Males weighing between 20 and 28 gm. and from 8 to 12 weeks old were used for irradiation and injection. The bone marrow and lymph-node donors were female mice 6 to 8 weeks old. Donor rats were 6-week-old females of the highly inbred WAG strain.[6]

All animals had access *ad libitum* to a commercial, cubed food and received supplements of cereals. The care of the animals and the specific pre-irradiation and postirradiation sanitary measures have been described elsewhere (*22*).

Irradiation.—The mice were irradiated in groups of 10 to 15 in a cir-

[5] These strains were obtained in 1953 from Dr. O. Mühlbock, of the Antoni van Leeuwenhoekhuis, Amsterdam, Netherlands. After this manuscript was completed, *H-2* typing of our CBA mice, performed by Dr. P. A. Gorer, London, showed that our CBA strain is not *H-2*[k] and is of an, as yet, unknown *H-2* type.

[6] Obtained in 1953 from Dr. W. F. J. Cuthbertson, of the Glaxo Laboratories, Greenford, Middlesex, England.

cular perspex cage. The physical factors of the X irradiation were as follows: 200 kvp, 20 ma., filtration 1.5 mm. of Cu, HVL = 1.8 mm. of Cu, distance to target 50 cm., dose rate 45 r per minute. The dosage was measured on the bottom of the perspex box with a Baldwin substandard ionization chamber. During the irradiation the dose was registered with a Baldwin dose meter (type Ionex) on the top of the box. The ratio between the two values was checked regularly. Maximal backscatter was used. The relation between dose and mortality of both mouse strains and of the F_1 hybrids has been published previously (*23*).

Cell suspensions.—Suspensions of bone marrow, spleen, lymph node, and thymus were prepared by squeezing the tissue through a nylon-gauze filter. Lymph node, thymus, and spleen were chopped with scissors before squeezing. Mouse and rat bone marrow were obtained from the femurs. Mouse lymph-node suspensions were prepared from inguinal and mesenteric lymph nodes, and rat lymph-node suspension from mesenteric lymph nodes only. The lymphoid cell suspensions were freed from fat droplets by centrifugation. Cell suspensions from several animals of the same strain were pooled when required, since the strains employed were highly inbred. The cells were suspended in Tyrode's solution. The total number of nucleated cells was counted in a hemocytometer. The percentage of living cells was estimated in an 0.2 percent solution of eosin in Tyrode's. Stained cells were considered dead. The cells were injected in a volume of 0.5 ml. into the tail vein, usually under light ether anesthesia. When two different suspensions were administered, 0.5 ml. of each was injected separately. Injection was performed within 4 hours after irradiation, except when indicated otherwise.

Autopsy.—At the beginning and end of each working day the cages were inspected for dead animals. Autopsies were performed immediately, or after storage of the dead animals in the refrigerator. The storage period did not exceed 24 hours. In spite of these precautions, *post-mortem* changes could not always be prevented. In some experiments animals were killed for autopsy at regular intervals after irradiation.

Histology.—Tissues were fixed in Bouin's solution. Paraffin sections of 7μ thickness were stained routinely with hematoxylin and eosin. Other staining techniques that were used will be mentioned with the results. In some instances, hematopoietic tissue imprints were made and were stained by the May-Grünwald-Giemsa method.

Bacteriology.—Blood was collected by heart puncture from nearly all mice that died before or on the 8th day after irradiation and from some mice that died after the 8th day. The samples were cultured on blood agar, Endo's medium, and glycerol agar.

Immunology.—CBA mice were sensitized against isoantigens of C57BL mice by transplantation of cells of a lymphosarcoma that originated spontaneously in one of our C57BL mice. About 1×10^6 cells were injected intravenously the first time. About 100×10^6 cells were injected intraperitoneally 7 days afterward in experiment 4 (table 7), and 13, 20, and 29 days afterward in experiment 5 (table 7). The mice were killed for

the collection of lymph-node cells, 15 days (expt. 4) and 8 days (expt. 5) after the last injection.

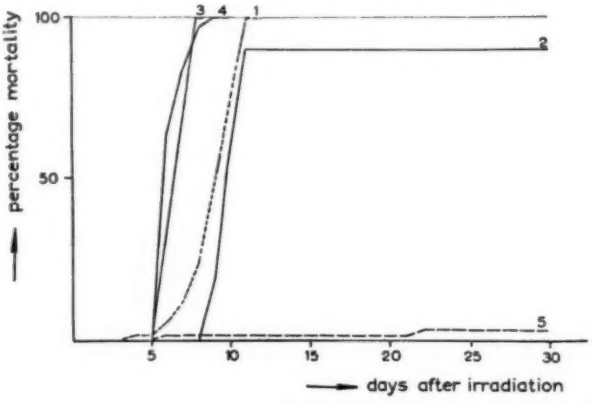
RESULTS

Irradiated Animals

Survival Times

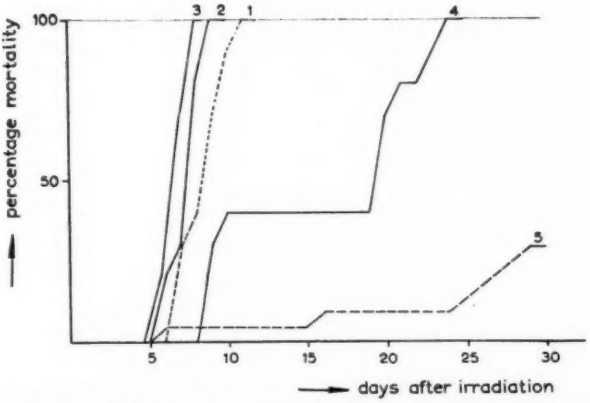
The number of bone marrow cells injected had been previously found to afford maximal 30-day protection (*23*). The injection of a large number of viable homologous lymph-node cells (1.6–5.8 × 10^6) shortly after the injection of homologous bone marrow cells to irradiated C57BL mice resulted in the death of more than 50 percent of the mice within 6 days, and of nearly 100 percent before the 8th day after irradiation and treatment (text-fig. 1). When homologous lymph-node cells were injected together with isologous bone marrow cells (curve #2, text-fig. 1), 8 of 9 mice died, but the mortality occurred a few days later.

The lethal effect of homologous lymphoid cells was also observed in CBA mice (text-fig. 2). The mortality cannot be accounted for merely by the fact that the cells were of lymphoid origin, since it was noted in another experiment that 5 × 10^6 isologous lymph-node cells had no lethal effect in C57BL mice that were irradiated with 700 r and treated with 10^6 isologous bone marrow cells.



TEXT-FIGURE 1.—Lethal effect of homologous lymph-node cells in C57BL mice. X-ray dose: 800 r.

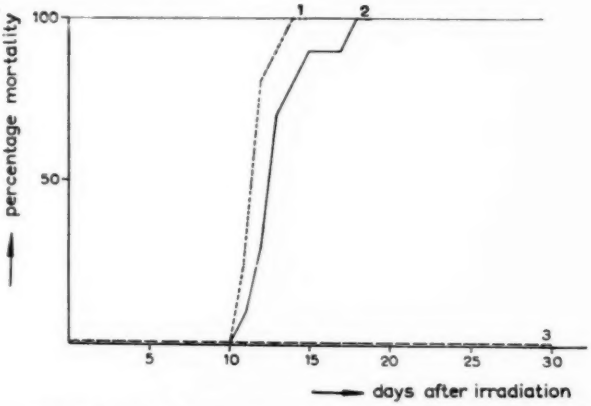
1. Controls (65 mice).
2. 1 × 10^6 isologous bone marrow cells + 5 × 10^6 CBA lymph-node cells (10 mice).
3. 5 × 10^6 CBA lymph-node cells (9 mice).
4. 12.5 to 25 × 10^6 CBA bone marrow cells + 1.6 to 5.8 × 10^6 CBA lymph-node cells (70 mice).
5. 12 to 25 × 10^6 CBA bone marrow cells (59 mice).



TEXT-FIGURE 2.—Lethal effect of homologous lymph-node cells in CBA mice. X-ray dose: 800 r.

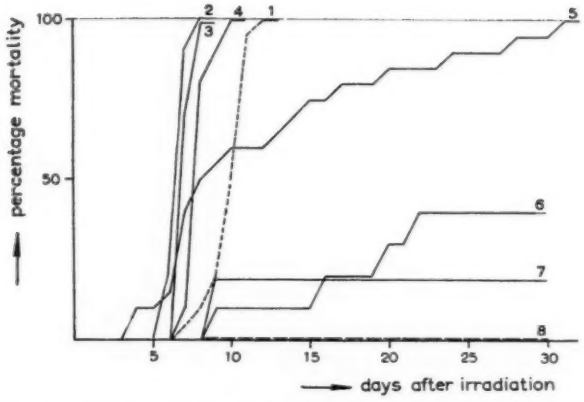
1. Controls (10 mice).
2. 1×10^6 isologous bone marrow cells + 5×10^6 C57BL lymph-node cells (10 mice).
3. 5×10^6 C57BL lymph-node cells (10 mice).
4. 25×10^6 C57BL bone marrow cells + 5×10^6 C57BL lymph-node cells (10 mice).
5. 25×10^6 C57BL bone marrow cells (22 mice).

The injection of only isologous lymphoid cells did not influence the survival of irradiated mice (*23*). A detrimental effect of isologous lymphoid cells was seen only if these cells were injected in combination with foreign bone marrow cells (text-fig. 3), which is in agreement with our previous observations (*24*).



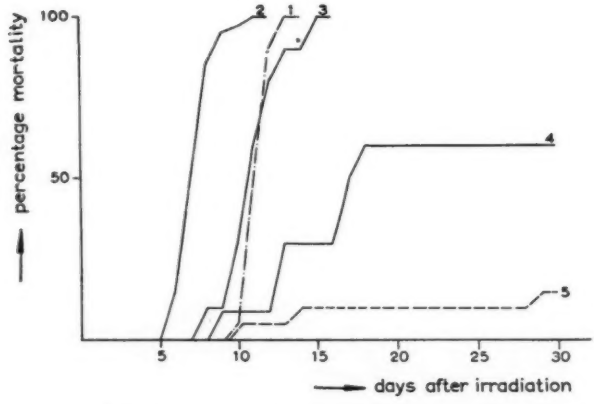
TEXT-FIGURE 3.—Effect of isologous lymph-node cells in CBA mice. X-ray dose: 675 r.

1. Controls (10 mice).
2. 25×10^6 C57BL bone marrow cells + 5×10^6 isologous lymph-node cells (10 mice).
3. 25×10^6 C57BL bone marrow cells (10 mice).



TEXT-FIGURE 4.—Relation between number of injected homologous lymph-node cells and survival time. X-ray dose: 800 r. Animals: C57BL males.

1. Controls (20 mice).
2. 12.5×10^6 CBA bone marrow cells + 3.2×10^6 CBA lymph-node cells (10 mice).
3. 12.5×10^6 CBA bone marrow cells + 1.6×10^6 CBA lymph-node cells (10 mice).
4. 12.5×10^6 CBA bone marrow cells + 0.8×10^6 CBA lymph-node cells (10 mice).
5. 12.5×10^6 CBA bone marrow cells + 0.4×10^6 CBA lymph-node cells (20 mice).
6. 12×10^6 CBA bone marrow cells + 0.2×10^6 CBA lymph-node cells (10 mice).
7. 12×10^6 CBA bone marrow cells + 0.1×10^6 CBA lymph-node cells (10 mice).
8. 12×10^6 CBA bone marrow cells.



TEXT-FIGURE 5.—Relation between number of injected parental strain lymph-node cells and survival in F_1 hybrid mice. X-ray dose: 800 r.

1. Controls (20 mice).
2. 1×10^6 CBA bone marrow cells + 0.4 to 10×10^6 CBA lymph-node cells (60 mice).
3. 1×10^6 CBA bone marrow cells + 0.2×10^6 CBA lymph-node cells (10 mice).
4. 1×10^6 CBA bone marrow cells + 0.1×10^6 CBA lymph-node cells (10 mice).
5. 1×10^6 CBA bone marrow cells (20 mice).

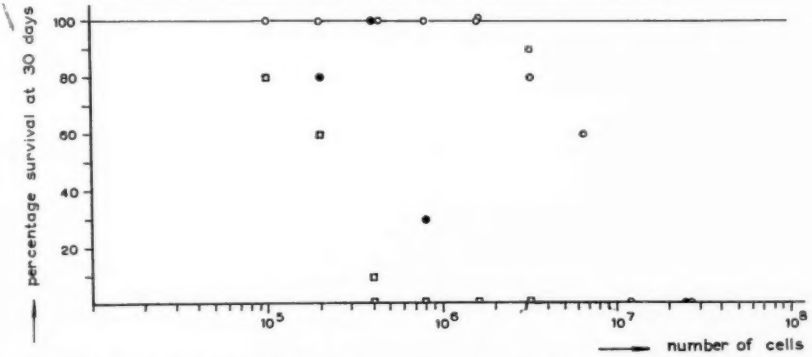
After the injection of a lethal number of homologous lymph-node cells into mice, irradiated and treated with homologous bone marrow, the survival time varied inversely with the number of lymphoid cells (text-fig. 4). A surprisingly small number of lymph-node cells was required to produce a lethal reaction. Lymph-node cells from mice of a parental strain had the same effect (or were even more effective) in F_1 hybrid mice as in mice of the other parental strain (text-fig. 5). A large number of lymph-node cells of F_1 hybrids injected together with F_1 bone marrow cells into CBA and C57BL mice caused no ill effect (table 1).

The effect of homologous lymphoid cells derived from different organs is illustrated in text-figure 6. Spleen cells were nearly as effective as lymph-node cells, but thymus cells were far less effective: about 30 times more thymus cells were needed to produce a comparable mortality.

TABLE 1.—Lethal effect of homologous lymphoid cells on mice irradiated and treated with bone marrow

Recipient strain	Donor strain and No. of injected cells ($\times 10^6$)		Number of mice	Thirty-day survivors*
	Bone marrow	Lymph-node		
F_1	CBA (1.0)	—	30	27
F_1	CBA (1.0)	CBA (0.4–19)	70	0
F_1	C57BL (1.0)	—	10	10
F_1	C57BL (1.0)	C57BL (20)	10	0
C57BL	F_1 (38)	F_1 (25)	10	10
C57BL	CBA (12–20)	—	59	57
C57BL	CBA (12–25)	CBA (0.8–5.8)	60	0
C57BL	C57BL (3.2)	—	10	10
C57BL	C57BL (3.2)	C57BL (5.0)	10	10
C57BL	C57BL (1.0)	CBA (5.0)	10	1
CBA	F_1 (38)	F_1 (25)	10	10
CBA	C57BL (25)	—	22	15
CBA	C57BL (25)	C57BL (5.0)	20	0
CBA	CBA (1.0)	C57BL (5.0)	10	0

*With a large number of 30-day survivors, it does not follow that the host-donor combination is a compatible one. In our experiments secondary mortality usually began after the 30th day post irradiation.



TEXT-FIGURE 6.—Lethal effect of homologous CBA lymphoid cells of different origin. Animals: C57BL males. Treatment: 800 r followed by injection of 12 to 12.5 $\times$ 10^6 CBA bone marrow cells + CBA lymphoid cells. □ = Lymph node; ● = spleen; ○ = thymus.

Some of the mice irradiated with a nonlethal dose of X rays (400 r) were killed by the injection of homologous lymph-node cells (table 2). Injection of the lymph-node cells 24 hours after irradiation is apparently more effective than injection after 3 hours. Lymphoid cells derived from mice that had been immunized against the host strain were even more noxious than lymphoid cells from normal homologous mice (*25*).

Surprisingly, heterologous lymph-node cells were observed to be considerably less effective than homologous lymph-node cells. Survivors at 30 and 60 days after irradiation did not differ significantly from those observed after treatment with rat bone marrow alone (table 3). However, when the rat lymph-node cells were injected 24 hours after irradiation, a marked lethal effect was noted (table 4). The effect of a great number of rat lymph-node cells injected 24 hours after irradiation was comparable to the effect of a small number of homologous lymph-node cells injected within a few hours after irradiation.

TABLE 2.—Lethal effect of homologous (CBA) lymphoid cells after nonlethal X-ray doses*

X-ray dose r	Interval between irradiation and administration of cells (*hours*)	Number of mice	Thirty-day survivors
100	3	14	14
	24	15	15
200	3	15	15
	24	15	15
300	3	14	14
	24	14	14
400	3	11	9
	24	15	0

*Recipients: C57BL males. Donors: CBA females. Each animal received 20×10^6 viable nucleated lymph-node cells intravenously.

TABLE 3.—Effect of rat lymph-node cells in rat bone marrow-treated (CBA × C57BL)F_1 hybrid mice exposed to 800 r X irradiation*

Number of injected lymph-node cells $\times 10^6$	Number of mice	Survivors at 30 days	Survivors at 60 days
0.0	10	10	3
1.6	10	8	1
3.2	10	10	1
6.4	10	9	2
12.8	10	10	1
25.0	10	10	0

*All mice received 25×10^6 rat bone marrow cells intravenously within a few hours after irradiation. The lymph-node cells were injected immediately after the bone marrow cells.

Clinical Observations

Mice that died during the first 5 to 10 days after irradiation and injection of homologous or heterologous lymph-node cells showed no distinct

TABLE 4.—Lethal effect of rat lymph-node cells in irradiated mice

Mouse strain	Radiation dose (r)	Rat bone marrow cells* injected after irradiation		Rat lymph-node cells† injected after irradiation		Number of mice	Number of survivors at 30 days
		Within a few hours	After 24 hours	Within a few hours	After 24 hours		
C57BL	800	+				20	16
C57BL	800	+		+		20	13
F_1	800	+				10	10
F_1	800	+			+	7	1
F_1	800		+			10	10
F_1	800		+		+	9	1
F_1	750	+				10	9
F_1	750	+			+	9	3

*Twenty-five to 49 × 10^6 viable cells.
†Five to 16 × 10^6 viable cells.

clinical features. Diarrhea and skin lesions were not observed during this period.

Mice that received foreign lymph-node cells and that died at 10 to 30 days after irradiation often showed a ruffled fur, loss of hair, and scaling of the skin. Wasting and diarrhea were observed after the 20th day. F_1 hybrid mice that were treated with parental-strain bone marrow only showed none of these symptoms (fig. 1), while in F_1 hybrid mice that were injected with parental-strain bone marrow and lymphoid cells the symptoms were obvious (fig. 2).

Bacteriological Observations

In spite of the fact that the cages were inspected for dead animals at the beginning and end of each working day, many of the mice showed *post-mortem* changes at autopsy. The possibility cannot be excluded that, in a few mice, the blood became infected after death. Still, in 45 of the 88 mice that died before the 9th day after irradiation, heart-blood cultures on blood plates were negative. In contrast, infection of the heart blood was noted in nearly 100 percent of untreated mice that died from the usual bone marrow syndrome (*26*).

Autopsy Findings

Mice irradiated with a lethal X-ray dose.—Four of 115 mice that were injected with homologous bone marrow and lymph-node cells and that died before the 9th day after irradiation showed hemorrhages, particularly in the stomach and the testis. Similar hemorrhages were observed in 5 of 6 untreated irradiated control mice. Hemorrhage was also common in many control mice of other experiments. Of the homologous lymph-node treated animals that died on the 9th to the 15th day, 2 of 22 showed hemorrhages, as against 17 of 20 control mice. The mice that died following treatment with homologous lymph-node cells were usually not anemic. In a few instances only a slight anemia was noted.

Macroscopically the lymph nodes were atrophic in all experimental

groups. After transplantation of homologous lymph nodes, the atrophy was less severe in some instances.

Control mice showed no typical skin lesions. In mice that were injected with homologous lymph-node cells, and that died before the 10th day after irradiation, a hyperemic skin was observed; mice that died at 10 to 30 days after irradiation showed a ruffled fur and scaling of the skin, particularly on the ventral side of the body. Loss of hair was observed usually on the dorsal side of the body and, in a few mice, a beginning ulceration was noted.

Calcification of the heart muscle was observed frequently in CBA mice treated with homologous bone marrow and in those treated with homologous bone marrow and lymph-node cells, but not in similarly treated C57BL mice.

In many mice from all experimental groups the lungs were congested and dark red.

Mice that died on the 10th to the 30th day after injection of heterologous lymph-node cells showed the same lesions as mice that received an injection of homologous lymph-node cells, and died during this period. F_1 hybrid mice treated with parental-strain lymphoid cells showed the same lesions as C57BL mice treated with CBA lymphoid cells.

Adequate information on the intestinal tract could not be obtained because of the frequency of *post-mortem* changes.

Mice irradiated with a nonlethal X-ray dose.—A lethal effect of homologous lymph-node cells was observed only in mice irradiated with 400 r. Small hemorrhages were seen in the stomach in 7 of 15 mice. In 1 mouse extensive focal necrosis was observed in the liver. In nearly all mice, the thymus, spleen, and lymph nodes showed various degrees of atrophy.

Hematological Observations

The recovery of the number of mononuclears in the peripheral blood occurred somewhat sooner after transplantation of homologous bone marrow and homologous lymph-node cells than after transplantation of homologous bone marrow cells only (table 5). After the transplantation of rat cells the recovery is apparently much more delayed, since low values (varying from 170 to 840) were noted on the 9th and 15th day post irradiation in mice treated with bone marrow only, as well as in animals that received rat lymph-node cells in addition. In the 4th week some animals reached values exceeding 2,000. The differences between the groups receiving bone marrow and lymph nodes and the groups receiving bone marrow only could not be evaluated satisfactorily because only a few mice were examined.

Histological Findings

Lymphatic and hematopoietic tissues.—Regeneration of the lymphatic tissues of animals that had received foreign bone marrow only was either absent or insignificant.

After transplantation of homologous lymph-node cells, regeneration was only slightly better; some minor regeneration was observed in most instances. Only one mouse showed an extensive recovery of lymphopoiesis in the lymph nodes, thymus, and spleen on the 7th day after irradiation and grafting. A complete restoration of the normal structure of these organs was never observed. The regeneration in lymph nodes and spleen was generally apparent by focal proliferation of lymphoid cells. In the thymus, regeneration was observed in the medulla of the lobules but not in the cortex.

A peculiar eosinophilic substance was observed in the spleen of one mouse injected with homologous lymphoid cells. This substance was located around a central artery and was separated from it by a highly edematous zone of reticular tissue in which a few plasma cells were seen. The eosinophilic substance showed a positive staining reaction with PAS,

TABLE 5.—Effect of injection of homologous (CBA) lymph-node cells on recovery of mononuclear leukocytes in blood of irradiated and homologous (CBA) bone marrow-treated C57BL mice*

Post irradiation day	Mononuclears per mm.^{3} of tail blood	
	Bone marrow only	Bone marrow and lymph-node cells
1	1060	873
2	144	285
3	192	143
4	137	102
5	212	293
6	466	1393
7	633	906

*X-ray dose to recipients: 800 r. Bone marrow dose: 25×10^6 cells. Lymph-node cells: 5×10^6. Each value in the table represents the mean of 3 animals.

methyl violet, and Azan staining methods, and a negative reaction with Congo red, van Gieson's and Hale's methods. Therefore it was concluded that this material consisted of fibrin or a closely related substance. As will be described later, this substance was also observed in nonirradiated mice injected with sensitized lymphoid cells.

The lymphatic tissues of mice that received an injection of rat lymphoid cells had the same appearance as those of mice that received an injection of homologous lymphatic cells.

Differences in the extent of lymphoid regeneration between mice treated with bone marrow and lymphoid cells and those treated with bone marrow cells only were difficult to evaluate. However, it is our impression that, in the former group, regeneration proceeds slightly faster.

In F_1 hybrid mice injected with CBA lymphoid and bone marrow cells, pyknosis and nuclear fragmentation of lymphoid cells in lymph nodes and spleen was found occasionally.

Swelling of reticular cells and a relative increase in number of reticular cells and sometimes of plasma cells were constantly noted in all experimental groups.

Erythropoiesis and granulopoiesis occurred in bone marrow and spleen of all mice receiving an injection of bone marrow cells but not in mice treated with lymph-node cells only.

Liver.—After treatment with homologous bone marrow and lymph-node cells, a limited number of necrotic foci, consisting of a few liver cells, were seen in the liver of 2 of 7 mice that were studied microscopically. In one other mouse degenerated liver cells were scattered throughout the liver. Livers of mice injected with heterologous lymphoid cells were not examined histologically.

Intestines.—Autolysis often made an evaluation of the changes in the mucosa very difficult. A loss of crypts was noted in the small and large intestines of many mice of all experimental groups during the first 14 days post irradiation. Slight edema of the submucosa was often seen. In a few animals that had received lymphoid and bone marrow cells, cryptitis was apparent by infiltration of granulocytes in individual crypts and in the surrounding tissue.

Skin.—In mice that died within 8 days after the injection of homologous lymphoid cells, no obvious skin lesions were observed, except for a moderate dilatation of blood vessels. Microscopic examination of affected areas of the skin of mice that received injections of rat bone marrow and rat lymph-node suspension revealed hyperkeratosis, slight parakeratosis, small areas of infiltration with granulocytes typically located in and directly beneath the stratum corneum, a loss of hair follicles, and a dilatation of blood vessels.

Heart.—No microscopic changes were observed in C57BL mice. In CBA mice a calcification of muscle fibers was often seen.

Lungs and kidneys.—Except for acute congestion no obvious microscopic changes were noted.

Nonirradiated Animals

To investigate whether or not irradiation is necessary for the induction of homologous lymphoid cell effects, some preliminary experiments were carried out with nonirradiated mice. The recipients were between 8 and 12 weeks old at the time of treatment. A survey of the results is given in table 6 and table 7.

In the first experiment the effect of lymphoid cells was investigated in a strictly homologous combination. Only one mouse died. At autopsy small lymph nodes and an atrophic spleen were noted. Microscopic examination revealed many small foci of necrosis in the liver and anaplastic lymphopoiesis in the thymus and the lymph nodes. Microscopically no pathological changes were found in the skin, intestines, pancreas, adrenal, testis, heart muscle, kidney, and spleen.

If the injected homologous cells do not kill the host within a few days after injection, a rejection of the grafted cells by the host seems likely.

TABLE 6.—Mortality of nonirradiated mice following lymph-node cell injections

Experiment No.	Recipients	Number of mice	Donor cells			Observation period (*days*)	Mortality	
			Origin	Bone marrow ($\times 10^6$)	Lymph node ($\times 10^6$)		Number of mice	Days after 1st injection
1a	C57BL	9	CBA	—	5	32	1	5
1b	C57BL	10	CBA	25	5	32	0	—
2a	F_1 hybrid	10	—	—	—	370	0	—
2b	F_1 hybrid	10	CBA	—	22	370	1	39
							1	174*
2c	F_1 hybrid	10	C57BL	—	22	370	0	—
3	F_1 hybrid	14	CBA	—	20–28†	250	2	72‡
							1	78
							1	84
							1	174
							1	188

*Killed by accident.
†Repeated injections on 1st, 8th, 14th, and 21st day.
‡Killed because of severe swelling of abdominal region.

TABLE 7.—Mortality of nonirradiated mice following lymph-node cell injections

Experiment No.	Recipients	Number of mice	Sensitized* donor cells			Observation period (*days*)	Mortality	
			Origin	Spleen cells ($\times 10^6$)	Lymph-node cells ($\times 10^6$)		Number of mice	Days after injection
4a	F_1 hybrid	3	CBA	—	26	47	2	14
							1	47
4b	F_1 hybrid	13	CBA	30	—	240	1	64
							1	70
5a	F_1 hybrid	16	CBA	—	20	190	0	—
5b	F_1 hybrid	24	CBA	40	—	190	2	14
							1	15
							1	17

*Derived from CBA mice immunized against C57BL isoantigens (*see* Methods).

Therefore the experiment was terminated on the 32d day after injection of the lymphoid cells.

Since the number of CBA lymph-node cells, which is required to produce mortality, is even smaller for irradiated F_1 hybrid than for irradiated C57BL mice, the parental strain-F_1 hybrid combination was used in a 2d experiment with nonirradiated animals. In the latter combination the host is not likely to produce an immunological reaction against the donor cells, so that long-term survival of the graft may be possible. The observation period was therefore extended.

Only one mouse died spontaneously on the 39th day after receiving an injection. Gross examination showed a small thymus. Microscopic examination was not performed because of *post-mortem* changes. In the mouse that died by accident on the 174th day, no pathological changes were seen at autopsy.

Because there was very little mortality in experiment 2, repeated injections of lymph-node cells were made in experiment 3. Within the first 2 months after transplantation no mortality was observed. Some diarrhea was noted in a few animals around the 30th day after transplantation. On the 72d day two mice were killed because of a swelling of the abdominal region. At autopsy severe edema of the subcutaneous connective tissue was observed. Organs of one mouse were studied microscopically. In the liver, many small foci of lymphopoiesis were seen. The bone marrow contained predominantly young blast cells. The red pulp of the spleen showed moderate hematopoiesis. In the lymph nodes the loss of the difference between cortex and medulla resulted in a monotonous picture. The tissue contained moderately large lymphoid cells that sometimes penetrated into the capsule. In the kidney and the intestines no lesions were observed. The pathological changes somewhat resembled those of leukemia in its initial stage. The relation to the lymphoid cell injections is not clear.

Four other mice died on the 78th, 84th, 174th, and 188th day, respectively, after transplantation. The first three mice showed severe *post-mortem* changes. The fourth had an enlarged heart and grayish-white, firm kidneys with a granular surface that microscopically showed chronic interstitial nephritis (glomerulosclerosis and a pseudothyroid structure).

In experiments 4 and 5 the lymphoid cells were derived from mice that had been sensitized previously against the isoantigens of the other parental strain. In experiment 4, two mice died on the 14th day after injection. At autopsy one showed a dilatation of the intestines, a small thymus, and a somewhat enlarged spleen. Microscopic examination was not performed. In the other mouse some clear fluid was noted in the thoracic cavity. Histologically atrophic crypts were seen in the colon. Lymphopoiesis in the spleen, lymph nodes, and Peyer's patches was severely depressed.

The mouse that died on the 47th day showed loss of hair and scaling of the skin, which began about 30 days after injection (fig. 3). At autopsy dilatation of the intestines was seen, the spleen and lymph

nodes were enlarged, the lymph nodes were hyperemic, and the thymus was atrophic. Microscopic examination was not performed.

In the group of mice treated with spleen cells, two animals showed diarrhea about the 30th to 60th day. One died on the 64th day. At autopsy many small, white patches were observed in the liver. Microscopically confluating necrotic foci infiltrated with leukocytes were seen in the liver. In the spleen, lymphopoiesis was much depressed. Another mouse died on the 70th day. Macroscopically and microscopically, similar lesions were noted in the liver.

In experiment 5 no diarrhea occurred. One mouse that died on the 14th day showed some loss of hair and atrophic hair follicles. The pathological changes in the mice that died on days 14 and 15 were similar. Two showed necrotic foci in the liver; in the third, similar foci were observed macroscopically. In the spleen, lymphopoiesis was severely depressed in one animal, and in all three mice the same eosinophilic substance was observed around the central arteries, which has been described earlier in an irradiated mouse that received homologous bone marrow and lymph-node cells. No pathological changes were observed in the sternal bone marrow and the thymus. The lungs were congested in all mice, and congestion of the kidneys was noted in two mice. The animal that died on the 17th day showed dark congested patches in the lungs. Microscopic examination was not performed.

It may be concluded that many of the symptoms and lesions resembled those of irradiated mice suffering from secondary disease following transplantation of foreign bone marrow only. This holds for the diarrhea, the skin lesions, the necrotic foci in the liver, the eosinophilic substance in the spleen, and the depression of the lymphopoiesis.

DISCUSSION

Since no adverse effect resulted from the injection of a large number of isologous lymph-node cells into irradiated mice treated with isologous bone marrow, or from the administration of F_1 hybrid lymph-node cells into parental strain mice, a direct toxic action of the lymphoid cells or their breakdown products seems unlikely. The detrimental effect of isologous lymphoid cells after they were injected into mice treated with foreign bone marrow is best explained by the assumption that the lymphoid cells proliferate and produce an immunological reaction against the bone marrow graft. This results in bone marrow aplasia and death, similar to the mortality observed in sublethally irradiated mice that received foreign bone marrow only (*23*, *24*).

The mechanism of action of homologous lymphoid cells appears to be mainly of an immunological nature. Theoretically both a host-antigraft and a graft-antihost reaction could be involved, especially so in the

nonirradiated animals. Using F_1-parental strain combinations, we have excluded the first possibility. The reaction of the homologous lymphoid cells is probably directed against all the host's tissues. Therefore the reaction of the host to the immunological attack by the donor lymphoid cells may be expected to be extremely variable. It is of particular interest that several of the symptoms and pathological changes resembled those encountered in mice suffering from secondary disease after foreign bone marrow transplantation.

It has been shown that the intensity of the reaction is related to the number of homologous lymphoid cells injected. A large number induces death within a week. A small number of cells may produce late death, presumably because the immunological graft-antihost reaction develops more slowly.

In nonirradiated mice the homologous graft may be expected to be rapidly destroyed except when parental strain cells are injected into F_1 hybrid recipients. In the latter combination, however, the proliferation stimulus to the grafted cells is probably weaker than in irradiated mice, and, in addition, the antigenic stimulus of the F_1 hybrids for antibody production by the parental strain cells might be less intensive than in the CBA-C57BL combinations. In spite of these apparently unfavorable conditions for the development of an efficient graft-antihost reaction, a certain percentage of the nonirradiated hosts was killed by the grafted lymphoid cells. The mortality was higher when sensitized homologous lymphoid cells were injected into F_1 hybrids. During the preparation of this manuscript, comparable results were reported by Cole and Ellis (*27*) and by Trentin (*28*, *29*).

Except for the severe diarrhea, all major symptoms and pathological changes noted in secondary disease after foreign bone marrow transplantation were observed separately or together in the nonirradiated recipients, described in this paper. This indicates that an immunological reaction of the graft against the host is of prime importance in the pathogenesis of secondary disease.

The specific localization of the lesions associated with a graft-antihost reaction, *e.g.*, in the skin, the liver, and the spleen, is at present poorly understood.

With regard to the activity of foreign lymphoid cells, our results have raised some additional problems:

1. Rat lymph-node cells were found to be far less effective than homologous mouse lymph-node cells. The administration of rat cells at 24 hours after irradiation was found to be decidedly more effective than immediately after the irradiation. This might indicate that rat lymphoid cells are more difficult to graft than homologous lymphoid cells.

The number of rat bone marrow cells and the number of homologous bone marrow cells required to protect irradiated mice are roughly equal, which points to a difference in transplantability between bone marrow cells and lymph-node cells. The assumption that lymphoid cells are more sensitive to the remaining immunological activity of the irradiated

hosts offers a satisfactory explanation for our observations, but additional information is definitely required before this can be accepted.

2. It was previously found that a small number (0.2×10^6) of isologous lymph-node cells prevented the therapeutical action of homologous bone marrow. The number of the isologous thymus cells required to obtain this effect is about 0.4 to 0.8×10^6 or 2 to 4 times that of isologous lymph-node cells (*24*). In the present study the minimal number of homologous lymph-node cells that is lethal to irradiated and bone marrow-treated recipients within 30 days after transplantation was estimated at 0.2 to 0.4×10^6. However, to induce death with homologous thymus cells, 4 to 8×10^6 or 20 times more cells were required. Although in both the isologous and the homologous cells, evidence for the presence of antibody-producing cells in the thymus has been obtained, the quantitative difference between the activity of thymus and lymph-node cells has not been explained.

Much additional information on the functional activity of immunologically active transplants is clearly needed before these problems can be solved.

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PLATE 7

FIGURE 1.—F_1 hybrid mouse 26 days after irradiation with 800 r and transplantation of 1×10^6 CBA bone marrow cells.

FIGURE 2.—F_1 hybrid mouse 26 days after irradiation with 800 r and transplantation of 1×10^6 CBA bone marrow cells and 1×10^5 CBA lymph-node cells.

FIGURE 3.—Nonirradiated F_1 hybrid mouse injected with 26×10^6 sensitized CBA lymph-node cells. Thirty-eight days after irradiation.

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The Pathogenesis of the Secondary Disease After Foreign Bone Marrow Transplantation in X-Irradiated Mice[1, 2]

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SUMMARY

The available information on the pathogenesis of the secondary disease after foreign bone marrow transplantation in irradiated mice is reviewed. Additional experiments are reported. The incidence of secondary disease after foreign bone marrow transplantation in mice was studied in various host-donor combinations after irradiation at three dosage levels. A characteristic secondary mortality was observed in CBA mice that received isologous bone marrow after the larger doses of X rays. Secondary mortality did not occur in F_1 hybrid mice that received isologous bone marrow, but severe diarrhea developed after the higher doses of X rays when followed by treatment with parental-strain bone marrow. In mice treated with rat bone marrow the development and persistance of symptoms of secondary disease was correlated with the presence of a functional rat bone marrow. Skin lesions and wasting, typical of secondary disease, as well as a high rate of mortality were observed in F_1 mice after the establishment of a parabiotic junction with a parental strain mouse of the same sex and age. Evidence has been provided in support of the hypothesis that the secondary disease in mice treated with foreign bone marrow is due to the combined effects of late radiation damage, an immunological reaction of the graft against the host, and an impaired immunological defense against microorganisms.—J. Nat. Cancer Inst. 23: 75–89, 1959.

IN MANY mammalian species the acute mortality which follows total-body X irradiation can be prevented by the administration of viable hematopoietic cells shortly after the irradiation [*see* review articles by Congdon (*1*) and van Bekkum (*2*)]. Most of the data concerning this therapeutical approach have been obtained with mice. When irradiated mice are treated with isologous bone marrow or spleen cells, the results are usually excellent. Acute (30-day) mortality can be completely prevented if the X-ray dose remains below the range at which intestinal damage is the principal cause of death. When the injection is made intravenously, the required number of viable, nucleated cells from isologous donors is amazingly small, namely, 10^5 for bone marrow and

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[2] Some of these data were presented at the Colloquim on Biological Problems of Grafting, March 18–21, 1959, at Liège, Belgium.

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10^6 for spleen (*3*). The results of follow-up studies after the 30th day have been unequivocal because mortality is negligible in some laboratories and considerable in others. However, the effect of the dose of radiation on long-term survival after isologous bone marrow treatment has not been investigated adequately. The available information from each laboratory is limited to one X-ray dose that approximates the LD100 for the particular strains employed. In this paper it will be shown that, in at least one mouse strain, a delayed mortality occurs after isologous bone marrow treatment if the X-ray dose is increased.

When foreign bone marrow—either homologous or heterologous—is used for treatment, the requirements and the eventual outcome prove to be quite different. The number of hematopoietic cells required for an equivalent protection is considerably greater than that of isologous cells. With intravenously administered bone marrow cells, this difference amounts to a factor of about 20 (*3*). In addition, foreign bone marrow treatment is effective only after radiation doses that approximate or exceed the LD100 minimum; after smaller doses the administration of foreign bone marrow may even be harmful (*4*, *5*).

The use of rat bone marrow to protect irradiated mice has made it possible to evaluate the functional state of the transplanted hematopoietic system at any time, by determining the origin of the erythrocytes and the granulocytes (*3*). In accordance with others, it was found that survival of the irradiated host mice was accompanied by colonization and proliferation of the donor cells. Failure to survive after median lethal doses of radiation is due to a secondary loss of the rat hematopoietic tissue (*6*). The latter reaction usually occurs during the 2d week after irradiation and will be referred to as the *acute rejection of the graft.* It is apparently caused by an immunological reaction of the host against the grafted tissue; the incidence of acute rejection of the bone marrow graft is dependent on the dose of radiation and probably also on the antigenic difference between the host and the donor (*7*). Our results with an F_1 hybrid-parental strain combination are in complete agreement with this viewpoint. Sublethally irradiated CBA mice showed 100 percent mortality when treated with C57BL bone marrow, only 65 percent mortality after treatment with (CBA ♂ × C57BL ♀)F_1 hybrid marrow. F_1 hybrid mice were completely protected by CBA bone marrow at all radiation dosages (unpublished results).

A delayed host antigraft reaction is presumably acting in rat bone marrow-treated mice that show a *gradual* return of host type cells with concomitant loss of the foreign bone marrow, after the latter has initially fully repopulated the tissues of the host. Animals that eventually regain their own hematopoietic system completely have been called "total reversals." In our experience, the reversing process is nearly always completed before the end of the 3d month after transplantation. Exceptionally the reversion remains incomplete for a very long time, during which the mice maintain a mixed population of host and donor-type blood cells (*8*). These mice will be called "partial reversals," while the term "true

chimeras" has been reserved for the mice that show donor-type cells only in the peripheral blood.

The above reaction may be classified as a *delayed rejection of the bone marrow graft.* The results of skin grafting experiments with these mice indicate a return of the host's immunological reactivity toward the donor's tissue (*9*).

It has been shown that the incidence of the delayed graft rejection is also dependent on X-ray dose; if the X-ray dose is increased the incidence of this reaction decreases (*10*).

Provided a proper X-ray dose and a sufficiently large number of foreign bone marrow cells have been administered, a satisfactory 30-day survival rate, up to 100 percent, and complete replacement of the host's hematopoietic system may be obtained. However, beginning in the 5th week, and in some experiments even a week earlier, the mice become ill and the majority die before the 100th day post irradiation. This *secondary disease* is clearly distinct from the acute radiation syndrome, with the peak of mortality during the 2d week, which develops in irradiated mice after lethal doses of radiation. The secondary disease is, in our experience,[4] characterized by three symptoms: emaciation, the passing of large amounts of loose feces (diarrhea), and dermatitis accompanied by loss of hair and a delay of the radiation-induced depigmentation of the fur. Since the characteristic secondary disease has only been observed after the transplantation of homologous or heterologous hematopoietic tissue (it has also been named homologous disease and foreign bone marrow disease), an immunological reaction between the host and the donor tissues has been postulated as the causative factor. Both antibody production by the host against the foreign hematopoietic tissue and by the graft against the host tissues have been proposed by various investigators (*see* the papers and discussions of the 1957 Gatlinburg Conference, *11*), Uphoff (*12*, *13*), and Trentin (*14*).

We have previously pointed out that although the basic cause of the secondary disease is probably a graft antihost reaction, some of the symptoms and even the ensuing mortality may well be due to a general decrease of the immunological defense reactions of the host animals (*2*). In addition late radiation damage has been postulated to be involved (*4*, *15*).

In this paper additional evidence will be presented to show that secondary disease is due to the single or combined action of the following factors: (*a*) an immunological reaction of the graft against the host; (*b*) late radiation damage; (*c*) a general weakness of the immunological defense system. In studying the nature of the secondary disease various approaches have been used.

The genetic approach.—This method has been employed by Uphoff (*12*, *13*), Trentin (*14*), and van Bekkum and Vos (*8*). The inability of F_1 hybrids to react against tissues of either of the parental strains is used to exclude the graft *versus* host reaction or the host *versus* graft reaction

[4] Based on experiments with host-donor combinations of mouse strains CBA, C57BL, and rat strain WAG.

from the experimental conditions, by grafting F_1 hybrid marrow into irradiated parental strain mice and *vice versa*.

In a series of experiments, Uphoff studied the occurrence of secondary disease in irradiated F_1 hybrids that received bone marrow transplants from one of the parental strains. A number of inbred strains and their F_1 hybrids were investigated. In every combination, except one, secondary disease developed, while the mice that received isologous (F_1 hybrid) bone marrow remained free of the disease. The one exception was provided by an F_1 hybrid whose parental strains (BALB/c and DBA/2) both possessed the *H-2*^{d} locus. The *H-2* locus is known to be the most important of the histocompatibility loci.

In irradiated parental strain mice receiving F_1 hybrid bone marrow, as well as in those receiving isologous marrow, some mortality occurred during the 2d and 3d month post irradiation, but this was due to amyloidosis of the kidney and a marked susceptibility to pneumonia (*13*), while typical secondary disease symptoms were not observed. Trentin reported a marked incidence of secondary mortality and secondary disease in irradiated F_1 hybrid mice after treatment with parental-strain bone marrow. In Trentin's paper (*14*), some of the groups of parental strain mice which received F_1 hybrid bone marrow showed significant mortality during the 2d and 3d month after irradiation, but in a subsequent paper this has been attributed to the use of very young mice as irradiated recipients (*16*).

Our own results again differed from those of the two previous authors. With CBA, C57BL, and (CBA ♂ × C57BL ♀)F_1 mice, none of the various F_1-parental combinations showed an appreciable incidence of secondary disease or mortality. The experiments described in this paper offer an explanation for this discrepancy.

In the present investigation it has been possible to differentiate between late irradiation effects and effects which result from an immunological reaction of the bone marrow graft against the host, by the use of higher doses of X rays followed by treatment with isologous or foreign bone marrow in F_1 and CBA mice.

Functional state of the bone marrow transplant in relation to the development of secondary disease.—It was mentioned previously that the condition of the graft can be evaluated satisfactorily after transplantation of *rat bone marrow* into irradiated mice. The incidence of partial and total reversals among the survivals of the first 100 days post irradiation was presented in detail elsewhere (*10*). Because these data contribute to an understanding of the mechanism of secondary disease, they will be briefly summarized. Of a group of 702 rat bone marrow-treated mice, which received a dose of 675 r (LD100), 126 survived the critical period of secondary disease. At the 100th day, 74, or 59 percent, showed a partial or complete recovery of the hematopoiesis of the host. At about 300 days after irradiation only 45 mice were living—15 of these were true chimeras, 8 were partial reversals, and 22 were total reversals. In contrast, from a group of 65 mice that received 800 r and rat bone marrow, 27 were living

after 100 days (2 partial reversals and 25 true chimeras). After 300 days only 5 mice had survived, all of which were true chimeras. Apparently, recovery of the host's blood-forming system is less likely to occur if the dose of radiation is increased. It has been our experience that the few mice that survive for a very long period, *e.g.*, more than 1½ years, were nearly all total reversals. Total reversals were always free of the skin lesions, which are characteristic of secondary disease. The incidence of these lesions is highest among true chimeras. These observations indicate that the presence of a functionally active graft is required for the maintenance of secondary disease.

Induction of secondary disease symptoms and mortality by the transplantation of foreign lymphoid tissue into irradiated and nonirradiated recipients, and by parabiosis.—In the preceding paper the administration of homologous lymph-node cells in addition to isologous or homologous bone marrow was reported to annihilate the beneficial effects of the bone marrow after total-body irradiation (*17*).

Evidence was provided to show that the grafted lymphoid cells react against the host animal. Furthermore, a disease resembling the secondary or foreign bone marrow disease was induced in F_1 mice after total-body irradiation (700 r) followed by the administration of parental-strain bone marrow and a small number of parental-strain lymph-node cells.

In nonirradiated F_1 mice some symptoms, closely resembling those found in the secondary disease, were evoked by the injection of lymph-node cells obtained from parental strain mice that had been sensitized previously against the tissues of the other parental strain.

In the present investigation similar symptoms, as well as a high mortality, have been observed in nonirradiated F_1 hybrids after parabiotic junction to a parental strain mouse. This technique allows the rather slow development of a one-directional immunological reaction between nonirradiated animals.

Information obtained from pathological and hematological studies.—The results of this part of our investigation will be published *in extensio*, but the main findings could not be disregarded in the present discussion.

In mice that died before the 30th day after foreign bone marrow transplantation, the bone marrow was the principal site of pathological changes, which is in agreement with the concept of a delayed rejection of the bone marrow graft, *i.e.*, a host *versus* graft reaction, occurring during that period.

After the 4th week, pathological changes were found predominantly in tissues other than the bone marrow, which excludes bone marrow failure by a host *versus* graft reaction, and which is compatible with the assumption of a graft *versus* host reaction being involved.

MATERIALS AND METHODS

Information on the experimental animals, the preparation of cell suspensions, and the irradiation technique has been provided in the preceding

article (*17*). Dose-mortality relationships for the mice used in this investigation have been published previously (*18*).

The LD50 was 575 r for CBA and C57BL mice, 625 r for the (CBA ♂ × C57BL ♀)F_1 hybrids. The LD100 was 650 r for CBA and C57BL and 675 r for F_1 hybrid mice.

Parabiosis, with the use of 6-week-old male mice, was done according to the technique of Bunster and Meyer (*19*). The operation was performed under sodium pentobarbitol (Nembutal®) anesthesia, supplemented by ether.

RESULTS

Secondary Disease and Mortality in Heterologous, Homologous, and F_1-Parental-Strain Combinations

The Effect of X-Ray Dose

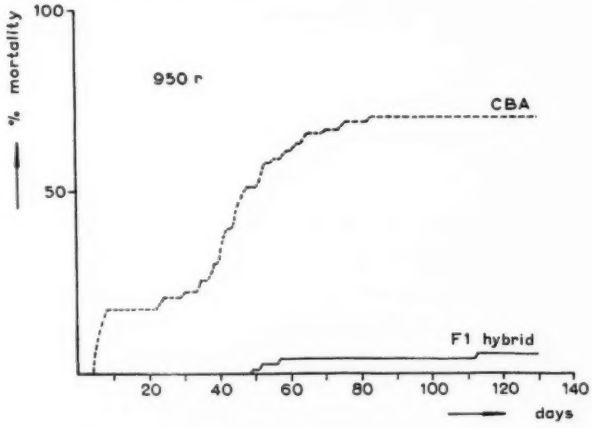
The number of bone marrow cells administered in these experiments was previously found to provide a maximal degree of protection in the 30-day survivals. (The number of cells was 10^6 when isologous and parental marrow were administered to F_1 hybrid combinations and more than 10^7—usually 2 to 3 × 10^7—when homologous and heterologous marrow and F_1 hybrid marrow were administered to parental strain combinations.) Table 1 shows that secondary mortality was extremely high in the strictly homologous and heterologous combinations. In these groups diarrhea was always severe, starting during the 4th week and usually subsiding after

TABLE 1.—Secondary mortality and diarrhea in various host-donor combinations after supralethal X-ray doses

Group	Recipient	Bone marrow donors	Irradiation dose in r	Number of mice	Percentage mortality at: 30 days	Percentage mortality at: 100 days	Diarrhea
1	CBA	Rat	675	504	40	80	Severe
2	CBA	Rat	800	85	9	66	Severe
3	CBA	Rat	950	10	40	100	Severe
4	CBA	C57BL	675	69	4	72	Severe
5	CBA	C57BL	800	22	32	100	Severe
6	CBA	C57BL	950	10	90	100	Severe
7	CBA	F_1 hybrid	675	30	0	0	Absent
8	CBA	F_1 hybrid	800	41	0	0	Incidental
9	CBA	F_1 hybrid	950	41	5	52	Slight
10	CBA	CBA	675	30	0	0	Absent
11	CBA	CBA	800	19	26	31	Incidental
12	CBA	CBA	950	89	22	71	Slight
13	F_1 hybrid	Rat	700–750	160	8	79	Severe
14	F_1 hybrid	CBA	700–750	110	0	0	Absent
15	F_1 hybrid	CBA	800	60	0	0	Slight
16	F_1 hybrid	CBA	950	70	12	20	Severe
17	F_1 hybrid	F_1 hybrid	700–750	180	2	2	Absent
18	F_1 hybrid	F_1 hybrid	950	70	0	4	Absent

the 12th week. Skin lesions were observed in the majority of these animals from the 5th week onward. In the animals that survived after the 100th day the skin usually remained abnormal.

After 675 to 750 r, secondary mortality was absent in both the F_1-CBA and the CBA-F_1 combinations, as well as after treatment with isologous marrow. However, secondary mortality was considerable in CBA mice after 950 r followed by *isologous* bone marrow injections. No symptoms characteristic of secondary disease were observed in these animals, although in a few cages loose feces were found occasionally. This disturbance lasted for a few days only and it never approached the severe form that was seen in groups 1, 2, 3, and 13 (rat bone marrow), 4, 5, and 6 (homologous bone marrow), and 16 (CBA marrow in F_1 mice). The highest secondary death rate occurred in the *isologous* CBA combination



TEXT-FIGURE 1.—Mortality rate of CBA and (CBA ♂ × C57BL ♀)F_1 hybrid male mice after radiation with a dose of 950 r followed by treatment with isologous bone marrow (10^6 viable, nucleated cells per mouse).

after 950 r between the 30th and the 60th day post irradiation (text-fig. 1), which is exactly the period of secondary death after homologous and heterologous bone marrow treatment. After treatment with isologous bone marrow, F_1 mice showed negligible secondary mortality and no secondary disease even after 950 r, which indicates that the F_1 mouse is much less sensitive to delayed radiation damage than the CBA mouse. After treatment with CBA marrow, some secondary mortality occurred in the 950 r group and diarrhea was noted in all cages from the 3d until the 7th week. In more than 50 percent of the cages the diarrhea was quite severe for some time, and the condition could not be distinguished from that seen after rat bone marrow treatment. In the 800 r group (# 15) diarrhea was less frequently observed and was milder in degree. Except in the mice treated with rat bone marrow, dermatitis was not obvious in any of the F_1 groups.

The results of the isologous bone marrow experiments show that F_1 mice were more resistant to late radiation damage than CBA mice. On the other hand, the incidence of the secondary mortality in F_1 and CBA mice was similar after rat bone marrow treatment indicating that: (1) Sensitivity of F_1 mice to the immunological reaction is similar to that of CBA mice. (2) Secondary death in F_1 mice is conditioned by the presence of foreign bone marrow.

The relative resistance of F_1 mice to parental-strain bone marrow may be due to a relatively small antigenic difference between the host and the graft, according to the gene-dosage concept, which was recently applied to this type of experimental data in a very elucidating paper by Uphoff and Law (*20*).

In this particular combination the mortality after 950 r and the diarrhea that developed after 800 and 950 r seem to be best interpreted as being at least partly the result of an immunological reaction of the graft against the host. The fact that the diarrhea was more severe in the 950 r group than in the 800 r group, as well as the occurrence of slight diarrhea in isologous bone marrow-treated CBA mice which were subjected to a dose of 950 r, shows that radiation damage is also involved in the development of this condition.

Attempts to Induce Secondary Disease Symptoms by Parabiosis Between F_1 and Parental Strain Mice

The establishment of a parabiotic union between F_1 hybrids and either one of the parental strain mice resulted in considerable acute mortality (table 2). The animals that died within 14 days have been grouped separately. In these cases, the condition of the junction was difficult to evaluate and part of this mortality has undoubtedly been caused by stress of the operation. However, it is our impression that the acute mortality is greater than in isologous twins, which might indicate that histoincompatibility is another factor involved.

In the mice that survived beyond the 14th day a perfect junction was established, except in a few animals that were killed and are excluded

TABLE 2.—Results of parabiotic union between F_1 hybrid and parental strain mice

Partners	Number dead before the 14th day:			Number dead after the 14th day:			Number living more than 90 days
	Parental	F_1	Both*	Parental	F_1	Both*	
CBA-F_1†	6	3	2	0	11	3‡	3
C57BL-F_1†	1	2	4	1	8	5§	2

*Both animals were found dead. It could not be established which one had died first.

†Deaths occurred at 17 to 62 days after the junction in the CBA-F_1 combination; at 20 to 97 days after the junction in the C57BL-F_1 combination.

‡In one mouse the CBA partner showed a large internal hemorrhage.

§In all 5 mice the C57BL partner showed a large internal hemorrhage.

from the table. In all but 1 of the 20 animals that could be evaluated properly, the F_1 hybrid partner was the first to die. In 8 mice it was not clear which one of the partners had died first. It is noteworthy that in 6 of these twins, the parental strain partner showed large abdominal or thoracic hemorrhages at autopsy.

The F_1 hybrid partner usually showed a progressive loss of weight resulting in a characteristic wasted appearance (fig. 1). At the time the junction was performed, the hybrid weighed a few gm. more than its parental strain partner of the same age.

In 5 twins the F_1 hybrid partner developed typical skin lesions consisting of loss of hair and ulceration (fig. 2). Diarrhea was never observed.

At autopsy a striking atrophy of the lymphoid organs and the wasted condition were the most prominent observations in the F_1 partners. No specific lesions were observed in the parental strain partners, except for the hemorrhages, when both partners were found dead simultaneously.

The results of the histological examinations will be published later, because additional experiments with irradiated parabionts are still in progress.

DISCUSSION

It now seems possible to present an analysis of the various pathological phenomena that follow the transplantation of foreign bone marrow in irradiated mice (text-fig. 2).

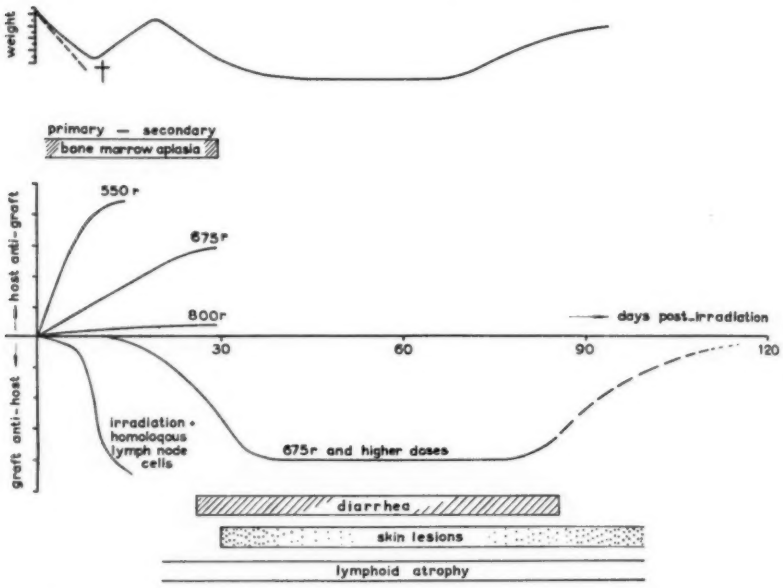
The initial recovery from the radiation-induced bone marrow aplasia is dependent on the degree of proliferation of the donor cells. The latter is in turn determined not only by the size of the transplant (number of cells) but also by the residual immunological reactivity of the host. After sublethal radiation, a proliferation of the grafted cells seems doubtful and early rejection is certain. After lethal doses of radiation and rat bone marrow transplantation, an initial proliferation of the donor cells may be followed by a secondary rejection, and even after a dose (675 r) exceeding the minimal LD100 some host animals regain the power to reject a fully established foreign bone marrow (total reversion). When the host's own hematopoietic tissues recover in time to take over, these reversals may survive. In the alternative case, death ensues and part of the mortality in the 3d and 4th week, after X-ray doses below the LD100, is due to this cause.

The characteristic sequelae of a graft rejection reaction can be summarized as: mortality with the highest rate before the 30th day; the absence of weight recovery; bone marrow aplasia; and, in the milder forms, a high proportion of "total reversals." After *800* r very few reversion reactions were observed and none proceeded to completion.

Recently we succeeded in identifying donor and host erythrocytes in the homologous combination and we have not found one single case of total reversal after 675 r (*15*). This indicates that the delayed host *versus*

graft reaction is also influenced by the degree of antigenic difference between the host and the donor. The results of Odell and Caldwell (*21*) are in excellent agreement with this point of view, since long-lasting bone marrow grafts were obtained between their closely related C and D rat strains after irradiation with an LD50.

Under conditions which favor the proliferation of the foreign cells (and maximally suppress host reactivity), the animals recover from the initial bone marrow damage and regain weight rapidly after the 10th day. However, about the 24th day a secondary weight loss occurs, accompanied by



TEXT-FIGURE 2.—Schematic representation of the relative importance of host *versus* graft and graft *versus* host reactions at various intervals after foreign bone marrow transplantation in irradiated mice. The hypothetical magnitude of the host *versus* graft and the graft *versus* host reaction are shown along the vertical axis in arbitrary units. The main pathological changes are put inside the horizontal bars on the corresponding side of the horizontal axis.

the first signs of diarrhea. These symptoms become rapidly more severe and at the end of the first month there is usually excessive diarrhea and emaciation. At this time skin lesions begin to appear. The majority of the animals die; the peak mortality is between the 30th and the 70th day. The prevalent pathological finding in this period is colitis. Many animals suffer from infections, such as pneumonia, and a few have focal degeneration of the liver. The lymphatic tissues are usually atrophic.

Evidence that the primary cause of the secondary disease is an immunological reaction of the graft against the host has been derived from experiments with F_1-parental strain combinations by Uphoff, Trentin,

and the present authors. The injection of homologous and heterologous lymph-node cells is lethal to irradiated mice under the conditions described in the preceding paper (*18*). The results obtained with F_1-parental strain combinations suggest that the latter mortality is caused by an immunological reaction of the grafted lymph-node cells against the host. In the animals that died between the 10th and the 30th day, after relatively low doses of lymphoid cells, clinical symptoms developed which resembled those of the secondary disease following foreign bone marrow transplantation. Skin lesions and diarrhea were observed in some of these animals. Apparently the transplantation of homologous lymph-node cells induces an accelerated development of secondary disease symptoms. Furthermore, every one of the characteristic symptoms of the secondary disease has been induced by the provocation of immune reactions directed against nonirradiated mice, either by lymphoid cell injections or by parabiosis. This accumulated indirect evidence in favor of a graft *versus* host mechanism being involved in the secondary disease has recently received additional support from the work of Feldman and Yaffe (*22*). These authors demonstrated the production of antihost agglutinins by lymphoid tissue derived from homologous radiation chimeras. In addition they presented evidence in support of a cytotoxic activity of the graft directed against the host.

However, radiation damage is also an important factor, which has been shown by the results obtained in heavily irradiated CBA mice that were treated with isologous bone marrow, as well as in irradiated F_1 mice after treatment with CBA bone marrow. These findings confirm suggestions made previously by Barnes and Loutit (*11*) and by ourselves (*4*).

The relative importance of the irradiation and the graft *versus* host reaction in the development of the secondary disease symptoms is dependent on the experimental conditions (table 3). Mortality can be caused by each of the two factors, skin lesions are mainly if not exclusively provoked by the immunological reactions, and the diarrhea is apparently the result of the combination of the immune reaction and the irradiation.

It should be pointed out that some host *versus* graft activity may persist after the 1st month as is manifested by delayed graft rejections; this may add to the complexity of symptoms, especially during the early period of secondary disease. Under experimental conditions which do not completely suppress the early host *versus* graft reaction, the pathological changes observed during the 4th to 6th week may be confusing, since lesions characteristic of both the host *versus* graft and the graft *versus* host reaction may be encountered (fig. 2).

Finally, there is evidence that a deficient immunological defense is of considerable significance in secondary disease. The results of skin transplantations (*5*, *9*, *14*) have shown that the nature of the transplantation immunity in radiation chimeras is determined by the antigenic composition of the foreign bone marrow graft. Taking into account all the other available evidence, the best explanation seems to be offered by the postulate that the immunological system in these radiation chimeras is

of the donor type. However, Brocades Zaalberg *et al.* have also observed that the transplantation reaction against skin, which is homologous with regard to the bone marrow donor as well as to the host, is usually abnormally slow (*9*). A severe atrophy of the lymphatic tissue is a characteristic finding in animals suffering from the secondary disease.

A high incidence of localized infections has been noted in mice suffering from secondary disease, in this as well as in other laboratories. Furthermore we have recently found that continuous treatment with an antibiotic (aureomycin) significantly inhibits the diarrhea and decreases the secondary mortality (*23*).

At present it is not known why the diarrhea and the mortality gradually diminish after the 70th day. In the groups treated with rat bone marrow this may partly reflect the relative increase of the number of total re-

TABLE 3.—Summary of experimental findings: factors involved in production of secondary disease symptoms

Treatment	Mortality (*secondary*)	Skin lesions	Diarrhea
Irradiation	+ Only after 950 r in CBA (isologous bone marrow)	−	+ Slight after 950 r in CBA (isologous bone marrow)
Foreign graft, *no* irradiation	+ Lymph-node cells (sensitized donors), parabiosis	+ Lymph-node cells (sensitized donors), parabiosis	+ Lymph-node cells (sensitized donors)
Irradiation and foreign bone marrow	++ Heterologous | do- Homologous / nors CBA into F_1 (950 r)	+ Heterologous | do- Homologous / nors	++ Heterologous | do- Homologous / nors CBA into F_1 (800 and 900 r)
Irradiation, foreign bone marrow, and lymph-node cells	+++ Heterologous | do- Homologous / nors CBA into F_1	+ Heterologous | do- Homologous / nors CBA into F_1	++ Heterologous | do- Homologous / nors CBA into F_1

versals, which no longer suffer from secondary disease. However this is certainly not the case in our groups treated with homologous bone marrow, in which total reversals did not occur. It should be recalled that the skin lesions usually do *not* subside after the 100th day except in the total reversals. In our experience most of the 100-day survivals that have retained the foreign bone marrow graft die within the 1st year after the irradiation.

It is possible that the chimeras which show a recovery from the secondary disease are the resistant individuals; the mortality sequence thus reflecting a selection process.

Another possibility seems that a graft-host adaptation gradually develops after the 70th day. The small percentage of survivors after this period constitutes a serious technical difficulty in elucidating this problem.

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PLATE 8

FIGURE 1.—Appearance of parabionts 28 days after operation.
Left: F_1 hybrid mouse with characteristic wasting.
Right: C57BL mouse. Dorsal skin of both partners shows residual damage from the depilating paste applied before operation.

FIGURE 2.—Appearance of parabionts 45 days after operation. Animal in *foreground* is an F_1 hybrid with characteristic wasting and skin lesions. Animal in *background* is a CBA mouse.

Mammary Tumors and Mammary-Gland Development in Virgin Mice of Strains C3H, RIII, and Their F_1 Hybrids [1, 2]

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SUMMARY

The incidence of mammary tumors was high in virgin females of strain C3H and in reciprocal F_1 hybrids between strains C3H and RIII, but comparatively low in virgin females of strain RIII. The incidence of tumors differed in the reciprocal hybrids, both, however, resembling strain C3H more than strain RIII. The mean tumor age in the (C3H × RIII)F_1 virgin hybrids was less than in either parental strain but more closely resembled that of C3H. The mean tumor age in the (RIII × C3H)F_1 virgin hybrids was greater than that in strain C3H and not significantly different from that in strain RIII mice. The differences in incidence of mammary tumors and in mean tumor age between the two hybrid groups suggest that the agent in the milk of strains C3H and RIII differs in quality or quantity, that of C3H being the more active. The mammary glands of strain RIII virgin females usually were not well developed, whereas the glands of strain C3H usually had many lateral buds or clusters of acini along the main ducts. The mammary glands of virgin hybrids between strains C3H and RIII resembled those of strain C3H virgin females. Frequently there was better development of acini in glands of hybrids than in those of strain C3H. No differences were observed in the extent of lateral budding and acinar development in the reciprocal hybrids.—J. Nat. Cancer Inst. 23: 91–107, 1959.

IN 1933, the staff of the Roscoe B. Jackson Memorial Laboratory (*1*) published the results of experiments utilizing reciprocal crosses between strains of mice with high and low incidences of mammary tumors. They found that F_1 hybrid females born to and raised by high-mammary-tumor strain mothers had a high incidence of mammary tumors, while those born to and raised by low-tumor strain mothers, although of identical genotype, were relatively free of tumors. Since this early work, many studies with high- and low-mammary-tumor strains of mice have shown that the maternal influence is an agent (virus) transferred in the milk of high-tumor strain females and present in many tissues of both sexes.

Few hybridization experiments utilizing strains with high incidences of mammary tumors have been reported. The results, although somewhat inconclusive and controversial, have tended to show that the milk

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agent from different strains differs in its ability to stimulate mammary-tumor development. Bittner (*2*) observed the tumor incidence in breeding females of strains A and D (DBA) and reciprocal hybrids between them. He found that the F_1 hybrid generation resembled the maternal strain and that mice of strain A and hybrids with strain A mothers had their tumors at an earlier age than did mice of strain D or hybrids with strain D mothers. Heston and Andervont (*3*) studied the development of mammary tumors in virgin mice of strains A and C3H and their reciprocal hybrids. They concluded that the difference in tumor incidence in the two parental strains was due to a difference in genic complex and that there was no conclusive evidence of a difference in the "inciting" agent. In a study of virgin females of the same strains and their hybrids, Bittner and Huseby (*4*) found that reciprocal hybrids differed both in incidence and age of appearance of mammary tumors. Hybrids with strain A mothers had more tumors earlier than hybrids with C3H mothers, a finding not in accord with that of Heston and Andervont.

Mice of strain A were reciprocally crossed with Marsh, another high-tumor strain, by Warner, Reinhard, and Goltz (*5*). F_1 hybrids with strain A mothers showed a higher tumor incidence than either of the parental strains and approximately double that of the reciprocal cross. Furthermore, the tumors appeared earlier in the hybrids with strain A mothers than in the parental strains or in the hybrids with Marsh strain mothers. They concluded that the milk agent was more concentrated in the A than in the Marsh strain, though the Marsh strain was more susceptible genetically to the milk agent.

In addition to comparing incidences of tumors in the A and C3H strains and their hybrids, Huseby and Bittner (*6*) made an extensive study of the comparative morphology of the mammary glands. They found that lateral buds were much less frequent at all ages in mice of strain A, which lacked adequate hormonal influence, than in mice of strain C3H and the F_1 hybrids. There were no histological differences in the glands of the reciprocal hybrids to correlate with the differences in the rates and times of tumor development.

We have crossed mice of strain C3H with those of another high-tumor strain, RIII, and have studied development of mammary glands and mammary tumors in virgin females of the two parental strains and the reciprocal hybrids. The RIII/Jax mice which we used resemble strain A mice in that virgin females have a low tumor incidence at a late average age. To determine the influence of the milk agent, mammary-tumor development in reciprocal hybrids between strains RIII and agent-deprived C3H ($C3H_eB$) was also observed. The results, presented in this paper, indicate that the milk agent in strains C3H and RIII differs in its effect on the incidence and age of appearance of mammary-gland tumors, but not on the morphology of the mammary glands.

MATERIALS AND METHODS

The strains of mice used in this study were RIII/Jax, C3H/Jax, and $C3H_eB$/Fe. Strains RIII and C3H harbor the milk agent and have high incidences of mammary tumors in breeding females. Mice of strain $C3H_eB$, derived from C3H ova transferred to the uteri of C57BL/6 mice, do not have the milk agent, and breeding females have a relatively low incidence of mammary tumors. We have found the incidence of tumors in breeding females of these strains to be as follows: 61.8 percent in RIII females (152 mice) at a mean age of 10.5 months; 94.1 percent in C3H females (170 mice) at a mean age of 8.8 months, and 25.7 percent in $C3H_eB$ females (268 mice) at a mean age of 20.0 months.

In order to study the effect of hybridization on the frequency and age of occurrence of mammary tumors in virgin females, reciprocal crosses were made between mice of strains C3H and RIII and also between $C3H_eB$ and RIII. In the designation of crosses in the text and tables, the maternal strain is listed first. The incidence of tumors and time of their appearance in the hybrids was compared to that in the parental strains. When mammary tumors were visible to the naked eye, the animals were killed by chloroform and a section of each tumor was taken for histologic diagnosis. Animals without visible mammary tumors were killed and autopsied when death from other causes seemed imminent.

In order to compare the structure of the mammary glands, the skins with adherent glands were removed from 250 mice, the majority of which were between 6 and 9 months of age. Third mammary glands were selected for study and whole mounts of these were stained with Mayer's hematoxylin.

OBSERVATIONS

Mammary tumors.—All tumors, including those in old agent-free mice, were diagnosed histologically as adenocarcinomas. Table 1 summarizes the data on incidences of tumors and mean ages when they occurred in virgin females of the C3H and RIII strains and their reciprocal hybrids. The percentage of virgin females with mammary tumors differed in the reciprocal hybrids ($X^2 = 9.2$; $P<0.01$) but each resembled the C3H strain (80.7%) rather than the RIII strain (16.4%). In the hybrid with the C3H mother (C3H $\times$ RIII), tumor incidence (97%) was higher than in C3H females ($X^2 = 14.2$; $P<0.01$), while in the hybrid with the RIII mother (RIII $\times$ C3H) tumor incidence (83.9%) was not significantly different ($X^2 = 0.03$; $P>0.05$).

Differences in tumor age were seen among some of the groups. The lowest mean tumor age was 8.5 months in (C3H $\times$ RIII)F_1 hybrids, compared to 12.2 months in C3H, 15.6 months in RIII, and 17.6 months in (RIII $\times$ C3H)F_1 hybrid mice. Differences between the following were found to be significant: C3H and the hybrid with the C3H mother ($t = 9.5$; $P<0.01$); C3H and the hybrid with the RIII mother ($t = 8.2$;

TABLE 1.—Occurrence of mammary tumors in virgin female mice

Cross ♀ × ♂	Number of animals observed	Animals with mammary-gland tumors			Animals dead without tumors		
		Percent	Mean age ± S.D.* (*months*)	Age range (*months*)	Percent	Mean age (*months*)	Age range (*months*)
C3H × C3H	119	80. 7	12. 2 ± 3. 5	6. 0–21. 2	19. 3	17. 0	9. 0–23. 0
C3H × RIII	101	97. 0	8. 5 ± 1. 6	5. 1–15. 1	3. 0	9. 1	8. 0– 9. 6
RIII × C3H	62	83. 9	17. 6 ± 4. 3	5. 7–27. 1	16. 1	19. 1	11. 3–24. 4
RIII × RIII	116	16. 4	15. 6 ± 3. 8	10. 3–23. 0	83. 6	20. 5	10. 0–31. 0
$C3H_eB$ × RIII	52	11. 5	20. 0 ± 2. 9	17. 8–25. 3	88. 5	23. 7	10. 5–28. 3
RIII × $C3H_eB$	45	84. 4	17. 3 ± 3. 4	8. 8–22. 1	15. 6	20. 7	17. 4–23. 0

*S.D. = Standard deviation.

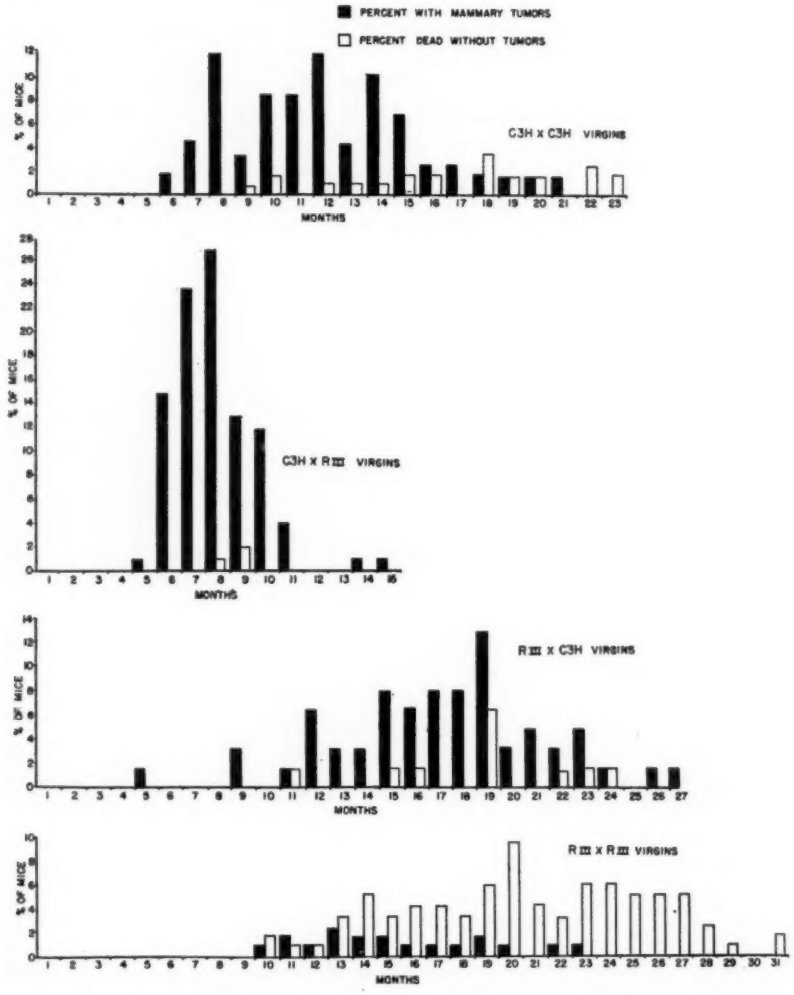
$P<0.01$); and the 2 hybrids ($t = 18.6$; $P<0.01$). There was no significant difference in mean tumor age between RIII females and hybrid females with RIII mothers ($t = 1.8$; $P>0.05$). Thus a marked maternal influence was shown in the ages at which tumors were observed in the 2 hybrid groups and by the fact that mean tumor ages in the hybrids were more like those in the maternal than paternal strains.

As expected, the incidence of mammary tumors in the females of crosses between strains RIII and C3H$_e$B depended upon the presence of the agent in the milk of the mother, incidence being high (84.4%) in the RIII × C3H$_e$B and low (11.5%) in the C3H$_e$B × RIII cross. There was no difference resulting from the presence of the agent in the paternal parent; the percentages of tumors in (RIII × C3H$_e$B)F$_1$ hybrids (84.4%), (RIII × C3H)F$_1$ hybrids (83.9%), and C3H (80.7%) were similar.

The mean tumor ages of the reciprocal hybrids between the RIII and C3H$_e$B were not different ($t = 1.3$; $P>0.05$), indicating that the agent in strain RIII milk influenced the incidence of tumors but not their early appearance. The agent in strain C3H milk, on the other hand, affected both incidence and early development of mammary tumors. The presence of the agent in the paternal parent did not affect the age of appearance of tumors in the hybrid, shown by the almost identical means (17.6 months with C3H father; 17.3 months with C3H$_e$B father). From these data it seems improbable that there was transfer of the C3H agent from the male.

The histogram (text-fig. 1) compares the proportion of tumors found at monthly intervals in virgin females of strains C3H, RIII, and their reciprocal F$_1$ hybrids. At 9 months of age, 80 percent of the (C3H × RIII)F$_1$ hybrids had mammary carcinoma, while at the same age, only 21 percent of the C3H mice and 5 percent of the (RIII × C3H)F$_1$ hybrids had mammary tumors. Among the 9-month-old RIII virgin females not a single mammary tumor was observed. The histogram also shows the percentage of mice dying without mammary tumors in each age period. It is noteworthy that, although 27 percent of the RIII virgins lived beyond 24 months of age, no mammary tumors appeared after that age. In the (RIII × C3H)F$_1$ virgin females, tumors did develop in mice over 2 years old.

Mammary-gland development.—Whole mounts were made of third glands of many mice of the inbred strains and their hybrids. The majority of these mice was between 6 and 9 months of age and many of them had mammary tumors. For purposes of comparison, mammary-gland development was arbitrarily graded as follows: 0, lateral buds absent or scarce; 1, many lateral buds along the main ducts but acini scarce or absent; 2, small clusters of acini; 3, larger clusters of acini; 4, profuse acinar development. Table 2 gives the percentage of glands in each grade. In the RIII mice there were few or no lateral buds in 50.0 percent of the glands, and clusters of acini were seen in the glands of only 3 mice. In the C3H mice small clusters of acini were seen in 54.9 percent of the glands, but only 5.9 percent showed profuse acinar develop-



TEXT-FIGURE 1.—Histogram showing percentage of mammary tumors at the age in months when they occurred in virgin females of strains C3H and RIII and in F_1 hybrids between these two strains.

ment. The glands of (C3H × RIII)F_1 virgins were strikingly different from those of RIII and resembled those of C3H, though frequently the hybrid glands showed even better acinar development. The glands of (RIII × C3H)F_1 hybrids and also those of ($C3H_eB$ × RIII)F_1 hybrids, assumed to be free of the milk agent, were similar to those of (C3H × RIII)F_1 hybrids in the extent of lateral budding and acinar development.

There were usually hyperplastic nodules in the glands of tumor-bearing females of all groups. Nodules were observed in most of the glands of

TABLE 2.—Percentage of mammary glands in visually graded groups ranging from 0, which indicates few or no lateral buds, to 4, which indicates profuse acinar development

Cross ♀ × ♂	Number of glands observed	Mean age (*months*)	Percentage of glands in grade:				
			0	1	2	3	4
RIII × RIII	42	6.2	50.0	42.8	4.8	2.4	0
C3H × C3H	51	6.5	7.8	9.8	54.9	21.6	5.9
C3H × RIII	61	7.8	0	19.7	32.8	27.8	19.7
RIII × C3H	21	7.7	0	19.0	28.6	38.1	14.3
$C3H_eB$ × RIII	38	7.0	0	13.2	34.2	39.4	13.2

(C3H × RIII)F_1 hybrids which were killed between the ages of 6 and 9 months, when tumors occurred. In the (RIII × C3H)F_1 hybrids, nodules of varying sizes were seen in the glands of the tumor-bearing females; very small nodules were observed in the glands of 9 out of 13 females, 6 months old, which did not have tumors. In the ($C3H_eB$ × RIII)F_1 hybrids, small or medium-sized nodules were seen in the glands of 6 out of the 7 old, tumor-bearing females; very small nodules were in the glands of 4 out of 11 females, 9 months old.

The degree of lateral budding or acinar development is illustrated by photomicrographs. Third glands of RIII virgins 6 months old are shown in figures 1 through 4. Figure 1 shows very few lateral buds and represents grade 0, the type found in 50 percent of the RIII females. Figure 2 is a gland with numerous lateral buds along the main ducts (grade 1), also a common type. Occasionally glands with small clusters of acini (grades 2 and 3), as shown in figures 3 and 4, were observed.

Photomicrographs of third glands of (C3H × RIII)F_1 hybrids, shown in figures 5 through 8, are examples of visually graded groups 1, 2, 3, and 4. Figure 5 shows numerous lateral buds along the main ducts, figure 6, small clusters of acini, figure 7, larger clusters of acini, and figure 8, profuse acinar development. Hyperplastic nodules, present in most of the glands of tumor-bearing females, are illustrated in the glands shown in these figures.

Figure 9 is the photomicrograph of a third gland of a (RIII × C3H)F_1 hybrid (grade 4). The acinar development is similar to that in the reciprocal cross (fig. 8). Figure 10, the photomicrograph of a third gland of a ($C3H_eB$ × RIII)F_1 virgin, 9 months old, shows small hyperplastic nodules, and figure 11, the gland of an old, tumor-bearing female of the same cross, shows several medium-sized nodules.

Photomicrographs of third glands of C3H virgins are shown in figures 12 and 13, and are examples of visually graded groups 4 and 2.

DISCUSSION

The results of observing virgin female mice of two high-mammary-tumor strains, C3H and RIII, and the hybrids between them, showed that there

were differences between the reciprocal hybrids that could not be attributed to genetic susceptibility, and that a maternal influence was operative. The hybrid females tended to resemble the maternal strains in tumor rate as measured by the mean age at which tumors occurred and the age at which the greatest proportion of females had tumors. More of the hybrids with C3H mothers (C3H × RIII) had tumors at earlier ages than mice of either parental strain or hybrids with RIII mothers. There were as many of these latter mice (RIII × C3H) with tumors as in strain C3H, but the tumors occurred much later than in mice with C3H mothers. In tumor incidence, the (RIII × C3H)F_1 virgins resembled C3H; in tumor age, RIII. These data suggest that the agent present in the milk of strain RIII mice was less stimulating to early development of mammary tumors than the agent in the milk of strain C3H mice.

That the agent in the milk of strain C3H females was of great importance in mammary-tumor development in susceptible hybrids was substantiated by the observation that in hybrids deprived of the agent, as in the cross $C3H_eB$ × RIII, tumor incidence was very low (11.5%) and the mean tumor age very high (20 months). In animals of identical genotype (C3H × RIII) with the C3H agent, there was an incidence of 97 percent at an average age of 8.5 months. Furthermore, the almost identical tumor rates of (RIII × C3H)F_1 and (RIII × $C3H_eB$)F_1 hybrids indicated that transfer of the C3H agent by the male was of no importance. It was concluded that the maternal influence observed in the reciprocal hybrids was the result of different capacities of the agent in the milk of strains RIII and C3H to stimulate mammary-tumor development in susceptible mice.

The possibility that the milk agent might vary in concentration and/or activity has been suggested not only by hybridization studies but also by experiments in which the agent was transferred with lactating mammary-gland tissue, mammary-tumor extracts, and dried tumor tissue from different strains to test-animals with uniform genetic constitution.

Although Heston and Andervont found only a bare suggestion of a difference in the milk agent from strains A and C3H mice, Bittner and Huseby concluded that the agent in strain A milk was more active than that in C3H. Barnum, Ball, and Bittner (*7*) reported that lactating-gland tissue of strain A females could be diluted more and yet give a higher incidence of tumors than lactating gland from strain C3H females, and Warner (*8*) stated that strain A milk agent would produce approximately three times as much mammary cancer as that in the Marsh strain.

Others observed the strain A agent to be less active than that in other strains. Dmochowski (*9*), using dried tumor tissue, found that the agent-potency in 3 strains, RIII, C3H, and A, was in that order. Mühlbock (*10*) compared tumor extracts from 5 strains and found that the strain A extract had less activity than DBA, RIII, C3H, and White Label. The agent in the Jax strain RIII mice appears from our observations to be less potent than that in the strain RIII mice used by Dmochowski, Mühlbock, and others.

That the explanation for such diverse findings probably lies in the existence of mutant strains of mouse mammary-tumor virus is substantiated by the observations of Blair (*11*) who found a subline of strain A mice in which tumors appeared at a considerably earlier age than in other sublines. By a series of reciprocal crossing and foster-nursing experiments, she traced the difference to an alteration in the activity of the milk agent (Bittner virus) in one subline of mice.

The mean tumor age of (C3H × RIII)F_1 hybrids was lower than in C3H virgins, indicating that, in this instance, hybridization was important in the development of tumors. Andervont (*12*) states that studies on the influence of hybridization upon the susceptibility of mice to various types of malignancies have shown that, when mice of highly inbred strains of known susceptibility are crossed, it is not possible to predict the degree of susceptibility of the resultant hybrids. For example, he found that hybrids derived from crossing two low-mammary-tumor strains, C57BL and I, were more susceptible to the development of mammary cancers than either parental strain.

Huseby and Bittner, in their description of the architecture of the mouse mammary gland, attempted to correlate structure and the three primary factors required for genesis of mammary tumors: inherited susceptibility, adequate hormonal stimulation, and the milk agent. They found that lateral budding was not dependent upon the presence of the agent, but tended to be more extensive in mice with adequate hormonal stimulation. Our results agree in general with their conclusions. RIII/Jax virgin females apparently lacked adequate hormonal stimulation, since their mammary glands did not show extensive lateral budding, in spite of the presence of the milk agent. The hybrid virgin females apparently inherited the hormonal factor from their C3H parents, since their mammary glands had many lateral buds or acini.

A similar effect of hybridization on mammary-gland structure was shown by Richardson (*13*). She found that when RIII females were mated with males of the low-mammary-tumor strain C57L, the tumor incidence in the F_1 virgins was high (62.5%) when compared to that of RIII virgins. A study of the mammae of these hybrids showed that usually many lateral buds were present in the glands.

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PLATES

PLATE 9

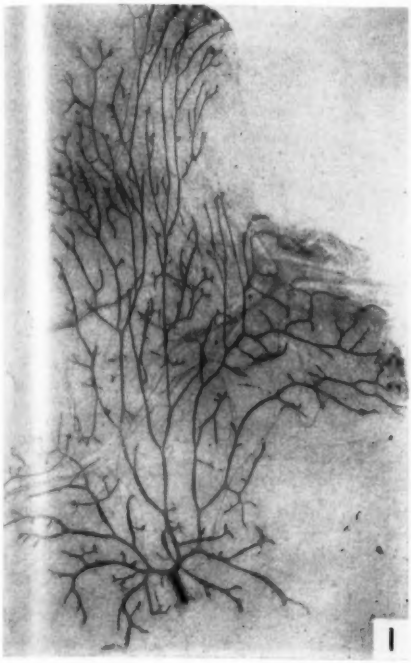
All figures are photomicrographs of third mammary glands of RIII virgin female mice 6 months of age.

FIGURE 1.—Gland showing few lateral buds (grade 0). This is an example of architecture found in 50 percent of the glands. × 6

FIGURE 2.—Mammary gland showing many lateral buds along the main ducts (grade 1). This represents architecture in over 40 percent of the glands. × 18

FIGURE 3.—Mammary gland showing small clusters of acini (grade 2). Glands of 2 out of 42 examined were like this. × 6

FIGURE 4.—Gland showing larger clusters of acini (grade 3). Glands developed to this extent were found in only one RIII mouse. × 18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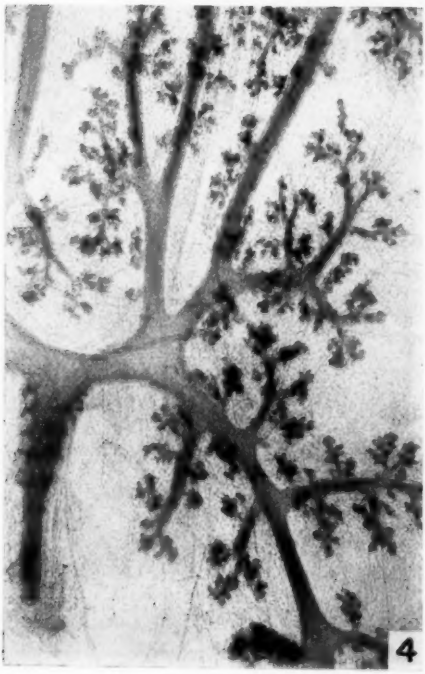
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PLATE 10

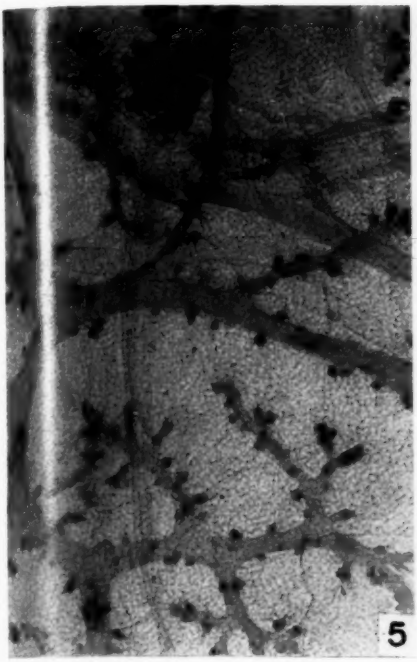
All figures are photomicrographs of third mammary glands of tumor-bearing (C3H × RIII)F_1 hybrid virgin female mice. *Note* hyperplastic nodules in these glands.

FIGURE 5.—Gland of female 9 months old (grade 1). One fifth of the glands was in this group. × 18

FIGURE 6.—Gland of female 7 months old (grade 2). One third of the glands was similar to this. × 18

FIGURE 7.—Gland of female 7 months old (grade 3). About one fourth of the glands was in this group. × 18

FIGURE 8.—Gland of female 7 months old, showing profuse acinar proliferation (grade 4). One fifth of the glands was similar to this. × 18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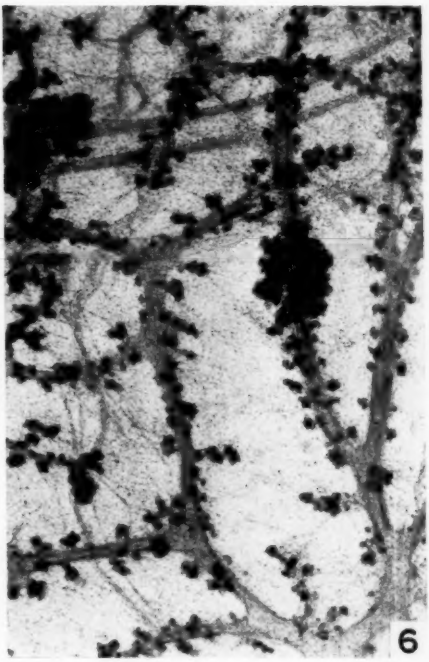
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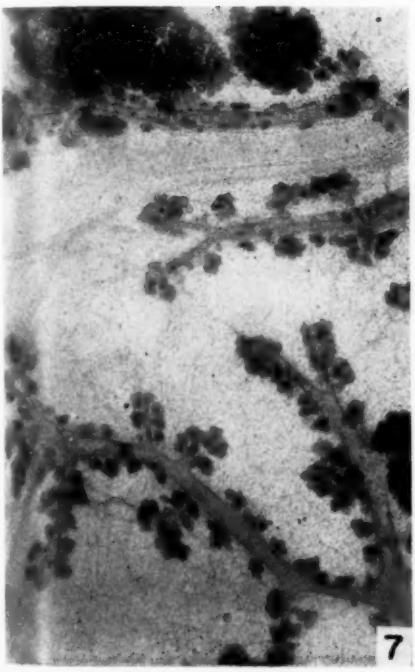
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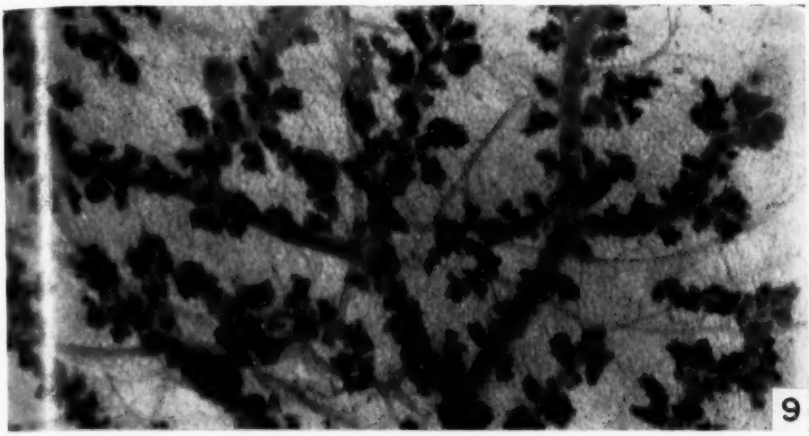
PLATE 11

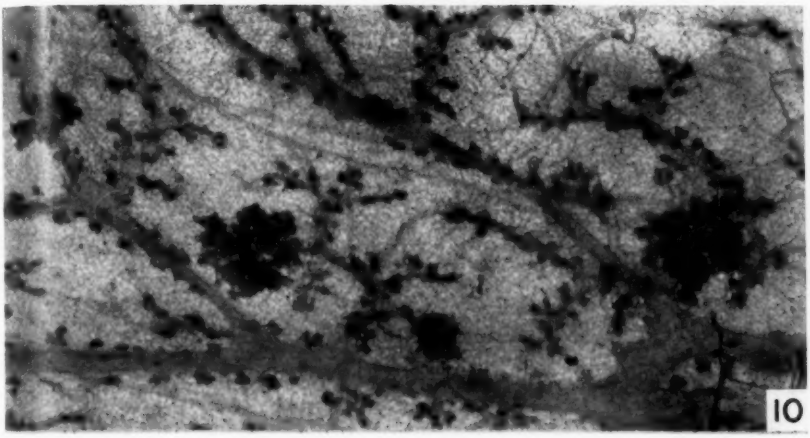
All figures are photomicrographs of third mammary glands of virgin female mice.

FIGURE 9.—Gland of (RIII × C3H)F_1 hybrid 6 months old (grade 4). × 18

FIGURE 10.—Gland of (C3H$_e$B × RIII)F_1 hybrid 9 months old (grade 2). *Note* small hyperplastic nodules. × 18

FIGURE 11.—Gland of tumor-bearing (C3H$_e$B × RIII)F_1 hybrid, 18 months old, showing medium-sized hyperplastic nodules. × 9





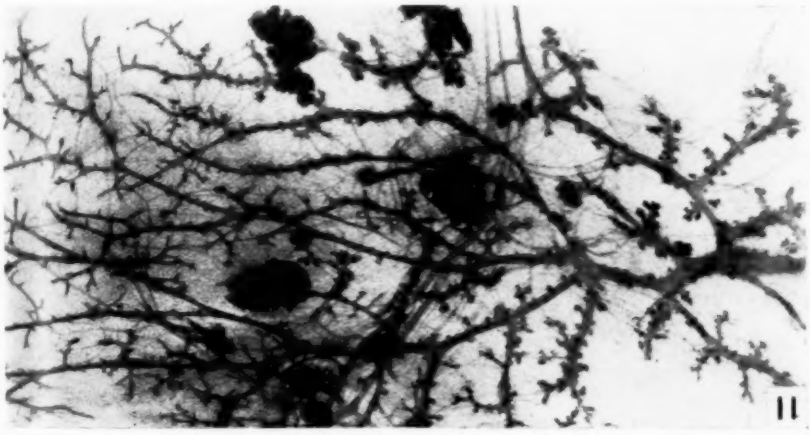


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PLATE 12

Both figures are photomicrographs of third mammary glands of C3H virgin female mice.

FIGURE 12.—Gland of female 6 months old (grade 4). Profuse acinar proliferation was less frequent in C3H than in the hybrids. × 18

FIGURE 13.—Gland of female 7 months old, showing small clusters of acini (grade 2). More than half of the C3H glands was similar to this. × 18

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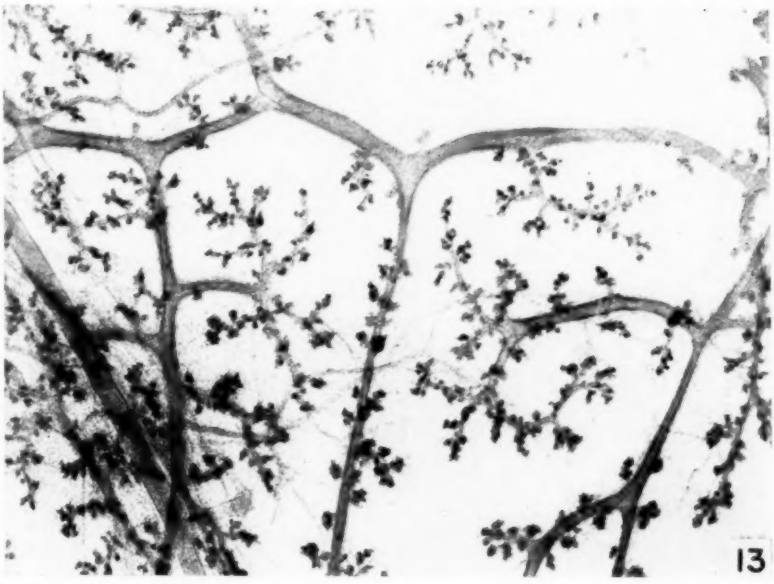
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Immunogenetic Studies on X-Irradiated Mice Treated With Hematopoietic Cells and Grafted With Tumor Tissues[1, 2]

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SUMMARY

A study was made of the behavior of tumor grafts in the radiation chimera to analyze the immune reactivity of the donor's hematopoietic cells and the influence on tumor cells of the radiation chimera. The regression of homografts of Sarcoma C10 and Sarcoma MC1M (of C3H origin) in irradiated C57BL animals treated with C57BL cells and their progressive growth in irradiated animals treated with C57BL fetal hematopoietic cells gave evidence of the ability of donor cells to form transplantation immunity against foreign antigens, thus substantiating the graft *versus* host theory as the basis for "homologous secondary disease." Tumor grafts produced metastases in irradiated animals, and irradiation and immunogenetic factors were shown to influence their development. The transplantation of Sarcoma SBL1 into various types of genetic chimeras was accompanied by immunogenetic changes in the tumor, which were manifested by the loss of strain specificity. The effect of total-body irradiation of the host and the absence of isologous C57BL cells were shown to be the main factors determining this change. Their analysis provided definite indications that the acquired homotransplantability of SBLx sublines is basically a result of immunogenetic transformation induced by the host and not a result of selection of pre-existing compatible cell variants.—J. Nat. Cancer Inst. 23: 109–131, 1959.

THE INOCULATION of X-irradiated animals with spleen or bone marrow cells from homologous donors resulted in colonization of the hematopoietic tissues of the irradiated recipient by the genetically "foreign" cells (*1*, *2*). Consequently, if homologous donor cells repopulate the antibody-forming organs of the irradiated animal, then theoretically the antibody-forming cells of the "homologous radiation chimera" can be stimulated immunologically by the isoantigens of the host. The transplantation immunity, which could thus be produced against the host's tissues, was postulated to be the main factor in the lethal "secondary disease" of homologous chimeras. Indirect evidence in support of the graft antihost response as the basis for incompatibility of radiation chimeras was derived mainly from experiments in which various hybrid combinations of hosts and donors were employed (*3–6*), and a direct

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approach to the analysis of the graft antihost response has indicated the dominance of this immune mechanism in determining the fate of irradiated mice treated with homologous hematopoietic cells (*6*). Thus, the inoculation of embryonic hematopoietic cells that (*a*) are not active immunologically and that (*b*) can develop immunological tolerance to foreign isoantigens might suggest a method for avoiding the immune response against the host's tissues (*6–8*). Transplantation tests on irradiated animals challenged with tumor homografts of known genetic markers might be useful in analyzing the immunological reactivity of donor cells. The level of transplantation immunity produced by the inoculated spleen cells against the tumor tissues might substantiate the role of the graft antihost response in homologous radiation chimeras, and the results obtained by the inoculation of fetal cells might indicate the degree of immunological tolerance that could be expected from the various types of treatment with genetically foreign cells. This approach has been undertaken in the present study.

The fate of tumor cells in radiation chimeras has, however, another aspect of basic significance. The principle of biological protection following total-body irradiation of mice was applied with some promising success to leukemic (*9*) and lymphoma-bearing animals (*10*). The extension of this principle to the treatment of leukemic animals of genetically heterogeneous populations (*i.e.*, human) would certainly involve the introduction of homologous hematopoietic cells into irradiated recipients. The behavior of tumor cells in such "homologous chimeras" is therefore relevant in this connection. In our experiments on the behavior of tumor homografts in X-irradiated animals, we have observed that some tumors may undergo immunogenetic changes resulting in a loss of strain specificity. These changes seem to have been determined by the immunogenetic relationship between the tumor, the inoculated cells, and the irradiated host. The possibility of using various genetic combinations of these components makes the "radiation chimera" a most adequate system for analysis of the factors determining such tumor-cell transformations. This analysis was the second object of the present investigation.

MATERIALS AND METHODS

Mice.—The mice were of the inbred strains C3H and C57BL, which were obtained from the same source that we used previously (*6*), and C57BL/6, which was supplied from the inbred stocks of the Roscoe B. Jackson Memorial Laboratory. The mice were all between 90 and 110 days of age at the start of each experiment.

Irradiation.—The animals were irradiated in Lucite containers (each mouse in a separate compartment), with a target object distance of 50 cm. The mice were exposed to 800 r (12 days, LD100) of 200 kv., 15 ma., with a 1 mm. Al filter and an 0.5 mm. Cu filter, delivered at a dose rate of

30 r per minute. Approximately 18 to 19 hours after irradiation the animals received intravenous injections of either spleen cells or embryonic liver cells. The spleen cell suspension was prepared from spleens of 8-day-old animals by breaking up the tissue, which had been suspended in Tyrode's solution, in a Ten Broeck glass homogenizer. Each animal received an intravenous injection of 0.4 ml., containing the equivalent of three quarters of a spleen (25×10^6 cells). The fetal-liver cell suspension was similarly prepared from fetuses of 17 to 19 days' gestation and was injected in doses of 25×10^6 cells. The inoculation of both types of cells was made into the tail vein.

Tumors.—The following tumors were used in the transplantation experiments: (1) The ascites form of Sarcoma MC1M, produced by G. Klein (*11*). This is a semihomotransplantable tumor that gives successful homografts in the intraperitoneal, but not the subcutaneous or intramuscular, site of mice of foreign strains (table 1). The progressively growing MC1M homografts produce an intense immune response, manifested by the formation of (*a*) a high level of hemagglutinins, (*b*) transplantation immunity (*12*), and (*c*) cytotoxic antibodies activated by complement (*13*). (2) Sarcoma C10 produced in C3H mice by subcutaneous administration of 0.2 ml. of 1.5 percent benzopyrene and maintained by intramuscular transplantation in the same strain. This is a strain-specific tumor, which grows only in C3H hosts, and is rejected by mice of all foreign strains tested (table 1). (3) Sarcoma SBL1, which was similarly produced in our laboratory by benzopyrene injection in C57BL mice (*14*) and is also a strain-specific tumor (table 1).

TABLE 1.—The homotransplantability of MC1M, C10, and C57BL tumors

Tumor	Tumor's strain of origin	Site of inoculation	Host's strains tested			
			C3H *H-2K*	C57BL *H-2B*	BALB/c *H-2D*	Swiss *H-2S*
MC1M	C3H	Intraperitoneal	+*	+	+	+
		Intramuscular	+	−†	−	−
C10	C3H	Intraperitoneal	+	−	−	−
		Intramuscular	+	−	−	−
SBL1	C57BL	Intraperitoneal	−	+	−	−
		Intramuscular	−	+	−	−

*+ = Lethal take of tumor grafts.
†− = Regression of tumor grafts.

EXPERIMENTS

Immunological Reactivity of the Inoculated Hematopoietic Cells

In our previous work it was clearly demonstrated that "secondary disease" in X-irradiated mice treated with homologous hematopoietic

cells was due to the graft's reaction against the host's isoantigens (*6*). In those experiments the genetic incompatibility was studied by treating irradiated animals with homologous hematopoietic cells. The following experiments were designed to obtain direct evidence that (*a*) the grafted hematopoietic cells can produce a high level of transplantation immunity, and that (*b*) the inoculated cells can develop immunological tolerance to foreign isoantigens, if they are inoculated at the fetal stage. In these experiments both the irradiated recipients and the donor cells were of the same genetic constitution, but were challenged with a foreign-tumor homograft.

1) Two groups of C57BL/6 mice were irradiated and inoculated with isologous spleen cells and isologous fetal liver cells, respectively. Immediately after the cell injection, each animal was challenged with a subcutaneous inoculation of 15×10^6 MC1M sarcoma cells (table 2, groups 1 and 2). The tumors of the spleen-treated animals regressed completely in 7 to 10 days following transplantation, whereas 7 of 7 C57BL mice treated with C57BL fetal liver cells had progressively growing tumors 30 days after transplantation.

TABLE 2.—Immunological reactivity of inoculated hematopoietic cells from adult and fetal donors

Group No.	Tumor graft	Irradiated host	Origin of inoculated hematopoietic cells	Progressive tumor growth*	Metastasis
1	MC1M	C57BL/6	C57BL/6 spleen	0/11	−
2			C57BL/6 fetal liver	7/7	+
3	C10	C57BL/6	C57BL/6 spleen	0/10	−
4			C57BL/6 fetal liver	5/5	−
5	C10	C3H	C3H spleen	10/10	+
6			C3H fetal liver	10/10	+
7	SBL1	C3H	C3H spleen	18/20	−
8			C3H fetal liver	20/20	+
9	SBL1	C3H	C57BL fetal liver	16/16	+

*Thirty days after tumor transplantation.

2) Two additional groups of C57BL/6 mice were similarly irradiated and treated with isologous spleen cells and isologous fetal liver cells, respectively, and then challenged with a subcutaneous graft of Sarcoma C10. The results were (table 2, groups 3 and 4) that in all the spleen-treated mice the grafted sarcoma regressed completely within 10 days, whereas mice treated with fetal isologous cells had progressively growing tumor homografts.

3) A further test was made in which C3H mice were irradiated. One group was treated with C3H spleen cells, and the other with C3H fetal liver cells. All animals in these two groups were then challenged with isologous Sarcoma C10. The results were (table 2, groups 5 and 6) that in both groups the tumors had grown progressively.

The differences found between the growth of tumor homografts in mice

treated with fetal cells and in mice treated with spleen cells must be attributed to an immune response produced by the grafted hematopoietic cells. Had the host been the responding factor in the homograft reaction obtained, then both groups should have behaved similarly, since all recipients received the same X-ray dose. It is also clear from groups 5 and 6 that the inoculation of spleen cells *per se* does not produce any unfavorable conditions for growth of tumor grafts in irradiated mice, since C3H mice that were treated with C3H spleen cells and grafted with Sarcoma C10 showed a lethal take of the tumor isograft. It is therefore concluded that, through an immune response, the injected spleen cells rejected the tumor homografts. The fetal liver cells, although capable of protecting the irradiated isologous recipient, are incapable of producing transplantation immunity, and they, therefore, accept the tumor homografts, at least during the first 30 days.

The progressive growth of MC1M and C10 in C57BL mice treated with fetal liver cells differed from their growth in the isologous C3H mice treated with C3H cells. The growth of the former homografts was comparatively slow, and, at 30 days after transplantation, the tumor became necrotic. When these tumor homografts were then transplanted back to C3H mice, they gave lethal takes, but the latent period of these grafts was rather long. The necrosis of the successful tumor homografts might be a result of a slow and delayed immune reaction that could have been elicited in the irradiated recipient.

4) Two groups of C3H mice were irradiated and inoculated with isologous spleen cells and isologous fetal liver cells, respectively. The animals in both groups were then challenged with a subcutaneous SBL1 graft. The results were (table 2) that in *both* groups progressive growth of the tumor homografts occurred. Unlike the results obtained with MC1M and C10, the SBL1 grew not only in homologous hosts that had been protected by fetal cells but also in C3H animals that had been protected by spleen cells. Subcutaneous inoculations of SBL1 are generally rejected by normal, nonirradiated C3H mice (table 1), after 10 days of initial growth. Two assumptions may be made to explain the progressive growth of SBL1: (*a*) This tumor may have a higher degree of homotransplantability and can therefore resist the low level of transplantation immunity that the repopulated C3H spleen cells produce. (*b*) The sarcoma SBL1 cells, while growing under the particular conditions of irradiated homologous hosts, undergo some immunogenetic changes that enable them to grow in an incompatible environment. The first assumption *a priori* does not seem to apply to the successful homotransplantation of SBL1 in irradiated recipients. This tumor is generally rejected by hosts from all foreign strains tested (table 1) and is very susceptible to transplantation immunity. Furthermore, the MC1M sarcoma, which does show a high degree of homotransplantability, is still rejected by the inoculated homologous spleen cells of the irradiated recipient. An experimental analysis of the second assumption was therefore undertaken, which is described later.

Protective Effect of Spleen Cells Stimulated Immunologically by "Foreign" Isoantigens

In our previous experiments on the treatment of mice with isologous spleen cells, we reported that no complete protection was conferred on the irradiated recipient (*6*). The results obtained in our present experiments, in which "isologous chimeras" were challenged with homologous tumor grafts, are therefore of special significance. Twelve C57BL mice that were irradiated and inoculated with C57BL spleen cells, and then challenged with Sarcoma C10, *all* survived for more than 210 days (and are still alive). Similar results of complete protection were obtained when irradiated C57BL mice were inoculated with C57BL spleen cells and grafted subcutaneously with MC1M. It seems that the antigenic stimulation of inoculated spleen cells by foreign tumor homografts has elicited an augmented multiplication of these spleen cells, thus leading to a more effective repopulation of the hematopoietic tissues of the irradiated recipient. This augmented repopulation seems to have determined the complete protection obtained in these experiments.

Metastases

During experiments on the immunogenetic behavior of tumors grafted on irradiated hosts, we found that the growth of the tumor transplants involved the appearance of metastases. Progressively growing metastases in the inguinal, brachial, and axillary lymph nodes (fig. 1) were developed by the following tumors: MC1M grafted on irradiated C57BL mice that had been treated with C57BL fetal liver cells, C10 grafted on irradiated C3H mice that had been treated with either fetal or adult isologous cells, and SBL1 grafted on either C3H or C57BL mice that had been treated with fetal C57BL cells (table 2, groups 2, 5, 6, 8, and 9). The immunogenetic conditions in these experiments seem to have determined, to some extent, the formation of such metastases.

In experiments (table 2, groups 7, 8, and 9) in which SBL1 was implanted on irradiated C3H hosts, although the tumors grew both in animals treated with fetal cells and in animals treated with spleen cells, metastases developed only in hosts that had been protected by fetal cells. It seems that the infant spleen cells, although incapable of resisting the progressive growth of the original tumor homografts, did resist the development of metastases in lymph nodes of the host. It can be assumed that this resistance is not based on an immune mechanism, but depends on differences between spleen and fetal cells in their capacity to repopulate the lymph nodes. If the spleen cells produce a more efficient colonization of the lymphatic system, they might be expected to prevent the establishment of progressing metastases in these sites. However, the formation of metastasis by the C10 tumor seems to exclude this explanation. Transplants of C10 produced metastases in irradiated C3H mice treated with either fetal C3H cells or C3H spleen cells. In these instances, the repopulation by isologous spleen cells did not prevent the formation of metastases,

probably since the inoculated cells were of the same genetic constitution as that of the tumor. When, however, C57BL mice were irradiated and treated with isologous cells, the immune response was too weak to prevent the growth of the original implant, but it seems to have rejected the progressive development of metastases.

The MC1M tumor behaved like the SBL1 in its capacity to develop metastases in C57BL hosts that had been protected by C57BL fetal liver cells.

Subcutaneous or intramuscular SBL1 tumor grafts in normal nonirradiated C57BL mice never develop metastases that can be detected macroscopically. Hence, the production of metastases by tumor grafts growing in irradiated recipients seems to depend on conditions formed by total-body X irradiation and the following protective treatment. These conditions can theoretically either induce an active migration of tumor cells from the original implant to the lymph nodes, thus resulting in the development of metastases, or, if such migration takes place even in nonirradiated hosts, then the irradiation can enable the progressive growth of the tumor cells that normally settle in the lymph nodes but do not multiply there. In order to differentiate between these two possibilities, the following test was made: Seven normal, nonirradiated C57BL mice were challenged with subcutaneous SBL1 transplants. Twenty to 25 days after the tumor transplantation the animals were killed and the axillary and inguinal lymph nodes were excised, washed in saline, minced, and inoculated intramuscularly into other normal C57BL mice. All inoculations of the lymphatic tissue from tumor-bearing animals resulted in progressively growing tumors in the secondary hosts. It is thus indicated that in normal hosts implanted with subcutaneous SBL1 grafts tumor cells migrate and settle in the lymph nodes, but this is not followed by any detectable growth. The total-body irradiation, in affecting the lymphatic tissues, seems to have activated the growth process of metastasizing tumor cells. This activation may, in certain instances, radically determine the survival time of the irradiated recipients. In all experiments in which irradiated C57BL mice were protected by either fetal or spleen cells from isologous donors and challenged with Sarcoma SBL1, the animals died within 10 to 12 days after tumor transplantation. The cause of death seems to be the rapid progression of the tumor metastases in the lymphatic tissues, a progression which interferes with the protective repopulation of the inoculated cells.

Induced Changes in Immunogenetic Properties of Tumor Homografts

As suggested, the progressive growth of SBL1 in the irradiated homologous hosts could be attributed to the immunogenetic changes that might have been induced in the tumor cells while they were growing under the particular conditions determined by the total-body irradiation and to the foreign genetic background of the recipient. In order to test this possibility, animals were irradiated, treated with isologous hematopoietic cells,

and grafted with homologous tumors. The growing homografts were then transplanted to nonirradiated secondary hosts so that changes in strain specificity could be tested. The following results were obtained:

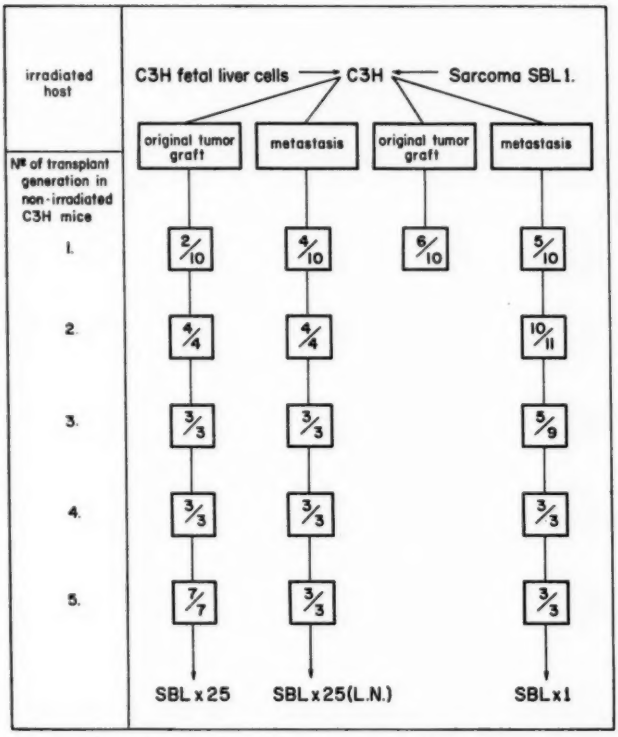
Sarcoma SBL1.—C3H mice were irradiated, treated with C3H fetal liver cells, and grafted with Sarcoma SBL1. After 27 days of growth, the original implant and a metastasis from 1 animal were transplanted to nonirradiated C3H and C57BL hosts. The results were (table 3) that not only tumors grafted to isologous recipients gave successful homografts but also tumors transplanted to homologous C3H recipients had a high percentage of progressive growth. In one experiment (table 3, expt. 1), 5 of 10 C3H hosts had progressively growing tumors at 20 days following implantation of the original tumor graft, and 6 of 10 C3H hosts implanted with the metastasis from the irradiated animal had successful homografts. There were similar results with a tumor from a second irradiated donor (table 3, expt. 2), in which 7 of 10 transplants of the original tumor and 7 of 10 transplants of its metastasis gave successful homografts. None of the 50 C3H control mice, grafted with SBL1 from nonirradiated C57BL, showed any sign of a tumor on the 20th day after transplantation. This tumor generally regresses in C3H mice on the 9th to 10th day after transplantation.

TABLE 3.—Growth of Sarcoma SBL1 in nonirradiated C3H animals following one transplant generation in irradiated C3H mice treated with C3H fetal cells

Expt. No.	Primary irradiated host	Inoculated hematopoietic cells	Origin of secondary graft	Secondary nonirradiated host	Number of tumor-bearing animals*	Number of lethal takes of tumor homograft
1	C3H	Fetal C3H	Original implant	C3H	5/10	2/10
				C57BL	10/10	10/10
			Metastasis	C3H	6/10	4/10
				C57BL	10/10	10/10
2	C3H	Fetal C3H	Original implant	C3H	7/10	6/10
				C57BL	10/10	10/10
			Metastasis	C3H	7/10	5/10
				C57BL	10/10	10/10
Control nonirradiated C3H					0/50	0/50

*Twenty days after tumor transplantation.

Tumors that gave successful homografts in the first transplant generation were then grafted successively in normal untreated C3H mice. In all experiments, homotransplantable lines of Sarcoma SBL1 that gave 100 percent lethal takes in C3H mice were established in the irradiated recipients after 1 transplant generation (text-fig. 1). These have been named SBL*x* sublines. It has thus been shown that one passage through lethally irradiated C3H mice inoculated with fetal C3H cells is sufficient to change the strain-specific SBL1 to a homotransplantable subline SBL*x*.



TEXT-FIGURE 1.—Establishment of homotransplantable SBLx sublines following transplantation of SBL1 in irradiated C3H mice treated with C3H fetal liver cells.

Usually, 2 transplant generations of the "transformed" tumor in nonirradiated C3H hosts resulted in a selection of homotransplantable tumor cells that gave 100 percent lethal takes in all successive transplantations. Although these tumors have acquired homotransplantability in mice of the C3H strain, the loss of genetic specificity was not strain specific: The SBLx sublines tested after 5 generations in normal C3H hosts gave successful homografts in the intramuscular and subcutaneous sites of all strains tested (table 4).

TABLE 4.—Homotransplantability of SBLx sublines

Strains	*H-2*	Site of transplantation	
		Intramuscular	Subcutaneous
C3H	*H-2^K*	10/10	10/10
BALB/c	*H-2^D*	8/8	8/8
Swiss	*H-2^S*	10/10	10/10
C57BL	*H-2^B*	10/10	10/10

MC1M and C10 tumors.—Similar experiments were carried out with MC1M and C10 tumors grafted on irradiated C57BL animals that had been treated with C57BL fetal liver cells, but no changes were obtained under the experimental conditions tested (*see* Discussion). Since Sarcoma C10 becomes very necrotic while growing in irradiated C3H animals, a test also was made in which Sarcoma C10 was implanted into irradiated C3H mice treated with C3H cells, *i.e.*, in which the C10 tumor was grafted in an isologous system and then tested for increased homotransplantability in nonirradiated recipients. No changes were, however, detected under these conditions (table 5).

TABLE 5.—Growth of MC1M and C10 tumors after one passage through radiation chimeras

Tumor	Primary irradiated host	Inoculated hematopoietic cells	Origin of secondary graft	Secondary nonirradiated host	Number of tumor-bearing animals	Number of lethal takes of tumor homografts
MC1M	C57BL/6	Fetal C57BL/6	Original implant	C57BL/6	0/10	0/10
				C3H	6/6	6/6
C10	C3H	Fetal C3H	Original implant	Swiss	0/6	0/6
				C3H	6/6	6/6
			Metastasis	Swiss	0/6	0/6
				C3H	6/6	6/6

Factors Determining Immunogenetic Changes of Sarcoma SBL1

Koprowski (*15, 16*) has shown that homotransplantability was induced in the strain-specific 6C3HED lymphoma and DBA lymphoma grown either in fetuses of a foreign strain (ICR-Swiss) or in infant mice of the same strain that have acquired immunological tolerance to the isoantigens of the tumor's strain of origin. In these experiments, as well as in our experiments on irradiated homologous hosts, two prerequisite conditions might have determined the induction of homotransplantability: (1) lack of strong immune response against the tumor grafts, and (2) growth of the tumors in a "foreign" antigenic background. The possibility of varying these two factors independently and of modifying each of them by the inoculation of various genetic types of hematopoietic cells makes the radiation chimera a most suitable system for the study of the factors involved in the induction of acquired homotransplantability.

The low immune reactivity of the fetal liver cells that repopulate the irradiated mice might be a decisive factor in the induction of homotransplantability. The fetal cells are probably incapable of producing an immune response when they are injected into the irradiated animals. They can, however, develop a gradual immune reactivity against tumor homografts during their maturation in the irradiated recipient. If the fetal C3H cells do not acquire complete immunological tolerance toward Sarcoma SBL1 homografts, they will develop a low immune reaction,

which might be involved in the immunogenetic changes induced in the grafted tumor.

To test whether these potentially responsive cells determine the immunogenetic changes in tumor cells, irradiated animals were used that had been treated with fetal cells isologous to the tumor homograft. The following experiment was performed:

Two groups of C3H mice were irradiated and treated, one group with C57BL fetal liver cells, *i.e.*, cells isologous to the tumor graft, and the other with C3H fetal liver cells, *i.e.*, cells homologous to the tumor graft. Each mouse was then challenged with a subcutaneous graft of Sarcoma SBL1. The tumors grew in both groups and developed large metastases in the lymph nodes. After 27 days of growth, the mice were killed and the tumors from each excised and transplanted intramuscularly to 5 nonirradiated mice. The tumor homografts were examined by palpation every 2 days. The criteria for the level of change in homotransplantability were: (*a*) period of growth in the C3H mice until regression, and (*b*) the maximal size that the tumor graft reached while growing in the homologous host. The results were (table 6) that transplants from all tumors that had grown on primary C3H mice treated with C3H fetal cells grew progressively and gave lethal takes, in varying degrees, in the secondary nonirradiated C3H hosts. Homotransplantable lines were established from almost all primary donors, and at least some tumors in every experimental group of secondary hosts reached the maximal size.

Transplants from tumors grown on irradiated C3H mice that had been inoculated with C57BL cells showed a lesser degree of homotransplantability (table 6)—only 11 of 30 grew in the secondary hosts for more than 20 days. Of these, only 6 resulted in lethal takes from which homo-

TABLE 6.—Intensity of change induced in SBL1 tumors by one passage through irradiated C3H mice treated with C3H or C57BL fetal cells

Tumor's primary hosts		Tumor-bearing secondary hosts		Maximal size reached by tumors				
Irradiated mice	Inoculated fetal cells	At 13 days	At 20 days	I*	II	III	IV	Lethal takes
C3H	C3H	5/5	3/5		1/5	3/5	1/5	1/5
		5/5	5/5		1/5		4/5	4/5†
		5/5	3/5			3/5	2/5	2/5†
		5/5	4/5		2/5	1/5	2/5	2/5†
C3H	C57BL/6	5/5	2/5		2/5		3/5	2/5†
		5/5	5/5		1/5	2/5	2/5	2/5
		5/5	3/5			3/5	2/5	2/5†
		0/5	0/5	1/5	4/5			
		3/5	1/5		4/5	1/5		
		1/5	0/5		5/5			
Control nonirradiated C57BL mice		0/5	0/5		5/5			

*I to IV = successive sizes of progressively growing tumors; I representing initial swelling of the implanted leg, II and III, intermediary stages, and IV, the final size at the "lethal stage."

†Homotransplantable SBL1 sublines established.

transplantable sublines were derived. They generally reached a smaller size than tumors from irradiated C3H mice treated with C3H cells. It should be stated, however, that the tumor grafts from irradiated C3H mice treated with C57BL fetal cells definitely increased their capacity to grow in an incompatible environment. All control grafts of Sarcoma SBL1 in C3H mice, which had not been transferred through an irradiated recipient, completely regressed by the 10th day. It seems, therefore, that even in the absence of a potentially immune reactivity of the repopulated hematopoietic cells, an increased tendency toward homotransplantability was conferred upon the tumor cells, though to a somewhat lesser degree than that obtained in the isologous chimeras.

In experiments reported in our previous paper (*6*), irradiated C3H mice, protected with fetal C57BL cells, that had survived for 60 to 180 days contained erythrocytes which were of purely C57BL origin. In these mice it seems improbable that an immune response can be formed against a tumor that is antigenically isologous to the blood cells of the host. However, these homologous chimeras are not completely protected by the genetically foreign fetal cells (*3–5*). This lack of general protection might be due to a delayed host *versus* graft reaction (*3*), *i.e.*, a reaction that immunologically affected the grafted SBL1 tumor in the last experiment. In the next experiment an attempt was therefore made to test the possible effect of the immune response produced by the host-type cells on the loss of strain specificity by the grafted SBL1. In these experiments (C3H $\times$ C57BL)F_1 mice were used as primary hosts for the SBL1. Such animals, containing the histocompatibility antigens of the C57BL strain, cannot respond immunologically against an SBL1 tumor. The following experiment was performed:

Two groups of (C3H $\times$ C57BL)F_1 mice were irradiated. The animals of one group were inoculated with C57BL fetal cells, the other with C3H fetal cells. Both groups and a third control group of nonirradiated (C3H $\times$ C57BL)F_1 mice were then grafted with Sarcoma SBL1. The tumors grew progressively in all the mice. After 27 days of growth, the tumors were excised and regrafted from each donor to 5 nonirradiated C3H mice. The results were (table 7) that no change occurred in the strain specificity of the tumors that had grown for 27 days in the nonirradiated F_1 mice, and that in the C3H hosts they gave only palpable homografts, which regressed completely between the 8th and 9th day. The tumors that grew for the same period in the irradiated animals grew progressively in the secondary C3H recipients until the 12th day, when differences appeared between the 2 experimental groups (table 7). The tumors from hybrid mice treated with C57BL cells then began to regress, whereas most of the tumors from hybrid animals treated with C3H cells grew until the 16th day, and nearly all of them reached a larger size than tumors from C57BL-treated mice. However, although in both groups a clear tendency for increased homotransplantability in C3H mice was definitely observed, we did not succeed in establishing homotransplantable sublines in either of them. Transplantation of the tumors to other non-

irradiated C3H animals, before their regression in the secondary nonirradiated hosts, resulted always in a complete rejection of the tumor homograft.

TABLE 7.—Intensity of change induced in SBL1 tumors by one passage through irradiated (C3H × C57BL)F_1 animals treated with C3H or C57BL fetal cells

Tumor's primary hosts		Tumor-bearing secondary hosts		Maximal size reached by tumors			
Irradiated mice	Inoculated fetal cells	At 13 days	At 20 days	I	II	III	IV
(C3H × C57BL)F_1	C3H	4/5	0/5		2/5	3/5	
		5/5	0/5		1/5	4/5	
		5/5	1/5		1/5	3/5	1/5
		5/5	0/5		2/5	3/5	
		5/5	1/5			5/5	
(C3H × C57BL)F_1	C57BL	4/5	0/5		5/5		
		1/5	0/5		4/5	1/5	
		2/5	1/5		3/5	2/5	
		0/5	—		5/5		
		0/5	—		5/5		
		0/5	—		5/5		
Control nonirradiated (C57BL × C3H)F_1		0/5	—	4/5	1/5		

From these experiments the following conclusions can be derived:

1) In all experiments on tumor transplantation in lethally irradiated mice inoculated with fetal hematopoietic cells, there was a change in the tumor cells toward an increased homotransplantability, which was detected by regrafting the tumor to secondary nonirradiated C3H hosts.

2) The intensity of this change showed a reverse correlation to the amount of the isologous component in the irradiated primary host in which this change took place. The order of increased homotransplantability seems to correspond to the following order of irradiated recipients: C3H⟶C3H>C57BL⟶C3H>C3H⟶(C3H × C57BL)F_1>C57BL⟶(C3H × C57BL)F_1.

3) The most intense change was produced in the most incompatible environment, *i.e.*, in irradiated C3H mice treated with C3H cells. However, since irradiated (C3H × C57BL)F_1 mice treated with C3H cells are probably more incompatible immunologically with SBL1 than are irradiated C3H mice treated with fetal C57BL cells, though the latter have produced a more intense change toward homotransplantability, it appears that the greater contribution of C57BL components in the irradiated hosts, and not the immunological conditions, tends to decrease the transformation in the SBL1. And accordingly, the lesser contribution of C57BL components tends to increase the transformation.

It is inferred, therefore, that two factors seem to interact in determining the conditions under which the hereditary loss of strain specificity can take place: (*a*) effect of irradiation (since the mere growth in F_1 or C3H during the same period does not change the strain specificity of SBL1); (*b*) absence of isologous antigens in the irradiated recipients.

DISCUSSION

Immune Reactivity of Donor Cells

Whether the genetic incompatibility found in homologous and heterologous radiation chimeras is due to a host antigraft response or to a graft antihost response is still a matter for controversy. The data obtained from some genetic combinations, in which F_1 hybrids were used as hosts and the parental strain as donors, and *vice versa*, seem to indicate that the incompatibility is based on the graft response against the host's antigens (*4–6*), but results obtained by other authors who used similar experimental principles with other F_1 combinations did not yield any clear evidence for either of these conceptions (*17*). A more direct approach to the immunological reactivity of homologous radiation chimeras was adopted in experiments designed to show that the donor cells are, in fact, active in producing a homograft reaction against the host's antigens, and that the graft antihost response is dominant in the secondary disease of radiation chimeras (*6*). Some very recent reports, by two groups of authors (*18*, *19*), on experiments in which classical immunological methods were used for the analysis of the graft *versus* host problem, claim to have provided evidence that the host's cells are the active element in antibody production in radiation chimera. However, in these approaches supporting the host antigraft theory, soluble antibodies were tested either with antihuman and antisheep red blood cells (*20*, *21*), or with rat γ-globulins (*21*). In the present study the activity of the donor cells in producing *transplantation immunity* was analyzed, since soluble antibodies produced in the homograft reaction do not appear to be decisive in transplantation immunity, even when their cytotoxic properties can be demonstrated (*13*), and it seems evident that if an immune reaction is involved in the "secondary disease" of radiation chimeras, it is of the type of transplantation immunity.

Main and Prehn (*20*) have shown that DBA mice protected by (BALB/c × DBA)F_1 cells can "take" skin grafts from BALB donors. Trentin (*4*) has similarly shown that C3H/CBA chimeras "took" skin grafts of both C3H and CBA mice. These experiments indicated that in these radiation chimeras the donor cells have colonized the antibody-forming host's tissues. The "acceptance" of the skin grafts reflects the existence of the donor cells, since these cells *do not* form an immune response against the skin graft. Similar tests with the use of tumor grafts (*21*, *22*) were performed with A/CBA chimeras. These have also shown that when the grafted tumors were isologous to the donor cells, the tumors will be "accepted" by the radiation chimeras. These data, although indicating that the donor cells have repopulated the irradiated recipient, do not, however, show that they can participate in the production of transplantation immunity. In further experiments aimed at analyzing the immune reactions taking place in radiation chimeras, it was found that when CBA mice were inoculated with CBA cells and grafted with Sarcoma I (SaI),

18 out of 36 gave successful homografts, whereas normal CBA animals generally rejected SaI homografts (*21*). The authors therefore concluded that "the radiation chimeras never fully recover their immunological response" (*21*). Furthermore, no evidence was presented to show whether the immune response in CBA chimeras that have rejected SaI was produced by the donor cells or by those of the host.

The experiments reported in the present investigation were designed to obtain an indication of the level of transplantation immunity produced in radiation chimeras and of the types of cells (donor's or host's) involved in its production. They have shown (1) that irradiated animals treated with spleen cells can produce an intense immune response. C57BL mice inoculated with C57BL spleen cells completely rejected grafts of Sarcoma C10 and Sarcoma MC1M, although the MC1M is a semihomotransplantable tumor. (2) It has been shown that the rejection of the tumor homograft was caused by the donor cells. When the donor cells were of fetal origin, the tumor gave successful homografts. The capability of the donor cells to form an intense homograft reaction supports the graft antihost theory. The progressive growth of C10 and MC1M in C57BL mice protected by fetal cells is attributed to the decreased ability of the donor cells to produce transplantation immunity. This is in accord with the possibility of avoiding "secondary disease" by treating irradiated mice with homologous fetal cells (*6–8*), which is based on the incapability of fetal cells to produce transplantation immunity and on the expected development in the donor cells of immunological tolerance toward the host's antigens. A similar tolerance should theoretically develop toward tumor homografts in isologous chimeras protected by fetal cells. It appears, however, that when C10 and MC1M tumors are implanted into C57BL mice treated with fetal C57BL cells, a delayed immune response is produced against the tumor homografts. But since this response developed 25 to 30 days following the inoculation of the fetal cells, there was no indication that the donor or the host cells were involved in its production.

Antigenic Stimulation of Cell Proliferation

The complete protection obtained when irradiated C57BL mice treated with C57BL spleen cells were challenged with MC1M or C10 homografts was attributed to the antigenic stimulation of the donor's spleen cells, which probably caused an augmented multiplication of the donor cells and consequently a more efficient colonization of the host's damaged hematopoietic tissues. We previously obtained complete protection by inoculating C3H mice with mixed populations of spleen cells from C3H and C57BL donors (*6*). This protective effect was also explained by the mutual immune responses between the inoculated C3H and the C57BL spleen cells. These immune responses cause the elimination of the C57BL cells and the survival of the C3H cells; the latter, stimulated immunologically by the C57BL antigens, undergo an augmented mitosis leading to a more

effective colonization of the host. However, the cells responsible for the protective colonization of the irradiated animal are probably not the anti-body-forming cells *per se*. Yet an immunological stimulation caused an increased protection. It is therefore suggested that the antigenic stimulation in the hematopoietic tissue of the grafted spleen elicits an augmented mitosis of stem cells, of which only a part differentiate toward antibody-forming cells, whereas others form other types of blood cells. These latter types might be involved in the protective effect.

An interesting instance of cell multiplication in radiation chimeras, which might also be explained by an antigenic stimulation, was reported by Uphoff and Law (*23*). They found that irradiated mice protected by homologous bone marrow showed a high incidence of malignant lymphomas. No such tumors appeared in irradiated mice treated with isologous bone marrow cells. It therefore seems that in these instances an immunological etiology might be involved in the induction of the lymphomas. The lymphatic cells were antigenically stimulated by "foreign" cell types of the chimera. This stimulation, although primarily intended for the antibody-forming cells, caused an augmented mitosis in the lymphatic tissue, which led to a malignant development.

Immunogenetic Transformations in Radiation Chimeras

The transplantation of SBL1 into irradiated C3H mice protected by fetal cells was accompanied by the induction of homotransplantability in this tumor. The fact that one passage of SBL1 through the irradiated recipients was sufficient to establish a homotransplantable subline suggests that the loss of strain specificity is not a result of a gradual selection of compatible cells from pre-existing cell variants, but rather an induction of immunogenetic transformation by the host's environment in the tumor cells. In this respect, the change in the histocompatibility requirements of SBL1 differs fundamentally from that obtained by Krebs (*24*) after serial passages of a lymphosarcoma for 7 years in foreign genotypes exposed to total-body X irradiation. The increased homotransplantability acquired in Krebs' experiments seems very likely a result of a long selection and gradual concentration of compatible cells. The analysis of the factors determining the changes of SBL1 in the radiation chimera provides further evidence that the change in our experiments is an induced transformation.

The induction, at will, of a loss of strain specificity was obtained by a number of experiments. Barrett and Deringer have shown that one transplant generation of C3H mammary carcinomas through F_1 hybrids of C3H and a resistant strain resulted in a change, which led to an increased percentage of takes in backcrosses of F_1 hybrids and the resistant parental strain (*25–27*). This observation was confirmed by Hauschka (*28*) and Klein *et al.* (*29*), who have shown that the increased homotransplantability could also be detected in F_2 animals. Another process of induced homotransplantability was described by Koprowski (*15, 16*). He

found that the inoculation of the strain-specific 6C3HED and DBA lymphomas into fetuses of ICR mice, or into infant ICR mice that have been made tolerant to the tumors' antigens, resulted in a complete loss of strain specificity by the two tumors.

The Barrett-Deringer effect is a unique phenomenon of immunogenetic change, since the increased homotransplantability is genetically specific and manifested only in either F_2 mice or backcrosses of F_1 and the resistant strain. On the other hand, the change obtained by Koprowski might seem similar, at first glance, to our effect. However, an analysis of the conditions that determine the changes in SBL1, in relation to those of Koprowski's phenomenon and the immunogenetic properties acquired by the two processes (*30, 31*), seems to rule out the possibility of a similar mechanism that is responsible for the induced homotransplantability in the two cases.

With various genetic combinations of donor cells, irradiated hosts, and tumor grafts, data were obtained which indicate that the preceding X irradiation of the host is important in increasing the homotransplantability of the SBL1 tumor. Transplantation of SBL1 into normal (C3H $\times$ C57BL)F_1 animals did not result in any changes in the behavior of the tumor in foreign strains, whereas tumors that were transferred through (C3H $\times$ C57BL)F_1 mice, protected by either C3H or C57BL cells, did show a definite tendency toward increased homotransplantability. The effect of irradiating the host cannot in this case be attributed merely to a suppression of the immune response, since (C3H $\times$ C57BL)F_1 mice cannot, theoretically, produce an immune response against a SBL1 tumor, and irradiated (C3H $\times$ C57BL)F_1 mice protected by C57BL cells do not differ immunogenetically from nonirradiated (C3H $\times$ C57BL)F_1 hosts. Yet the latter did not induce any signs of change, whereas the irradiated animals did.

It should be stated, however, that the effect of irradiation *per se* is not decisive in the induction of the immunogenetic changes in the tumors. Implanting the SBL1 sarcoma in various types of radiation chimeras has shown that the various genetic backgrounds determined different intensities of change. It has thus been found that the transfer of SBL1 in (C3H $\times$ C57BL)F_1 mice, protected by either C3H or C57BL cells, increased the survival time of SBL1 when it was grafted to normal C3H mice, but the tumor did not acquire complete homotransplantability. On the other hand, the transfer of SBL1 in C3H mice protected by either C3H or C57BL cells resulted in a complete loss of strain specificity. C3H animals treated with C3H cells induced a more intense change than C3H mice treated with C57BL cells. It may therefore appear that the more incompatible the chimera is to the tumor, the more intense is the change induced in the tumor cells. Thus, the "low" immune reactivity of the incompatible chimera might have participated in the mechanism of transplantation. However, a comparison of the intensity of the change obtained in the various systems of donor cells/irradiated hosts shows that the *absence* of C57BL cells probably is more important than the

immunological incompatibility. The lower the contribution of C57BL components in the radiation chimera, the greater the intensity of the change conferred upon the tumor cells, irrespective of the theoretical immunological reactivity against the tumor.

Neither the effect of total-body irradiation nor the absence of isologous cells explains, at this stage, the mechanism through which the strain-specific SBL1 changes its histocompatibility requirements. However, neither of these factors seems to exert a selective pressure that can differentiate between homotransplantable and nonhomotransplantable cells. Therefore, the possibility that the loss of strain specificity by SBLx sublines is a result of immunoselection of certain cell types from the original cell population seems to be ruled out. The change of SBL1 to SBLx is thus attributed to a change in the immunogenetic properties of the tumor cells, which is induced by the conditions prevailing in the radiation chimera. This conclusion is further supported by our study of the immunogenetic characteristics acquired by the adapted tumors (*30*).

Whether or not the adaptation of SBL1 in the radiation chimera is a general phenomenon must await further investigation. The negative results obtained with Sarcoma C10 and Sarcoma MC1M might be due to the too-short growth period of the tumor in the irradiated host. We have obtained some data on the SBL1 tumor, which indicate that a longer exposure of the tumor to the inducing chimera results in a more pronounced change. Experiments (unpublished) were also carried out with the 6C3HED lymphosarcoma, which is specific to the C3H strain. Its transfer through irradiated C57BL mice treated with C57BL fetal cells resulted in a change toward increased homotransplantability, which was manifested in a longer survival time of the tumor when grafted later to normal nonirradiated C57BL mice.

Ilbery *et al.* (*22*) described an experiment in which CBA /CBA chimeras were challenged with SaI, which was rejected by the animals. These mice were then grafted with BP8 (a tumor originating in C3H, but which also "takes" in CBA mice). One "peculiar" looking BP8 graft was then transplanted to C3H and A animals, and grew in *both* strains. It might be that in this experiment the BP8 has also undergone immunogenetic changes similar to those of SBL1.

Stimulation of Metastases in Radiation Chimeras

Tumors grafted on radiation chimeras developed large metastases, mainly in the lymph nodes. A similar phenomenon of metastasis was mentioned previously (*21*). In the present study it was shown that, when SBL1 is transplanted to normal nonirradiated hosts, tumor cells invade the lymphatic tissue and form emboli, but these never proceed to a metastatic development. The total-body X irradiation could have influenced the progressive growth of the metastasis, either by the damage caused to the lymphatic system which produced "physiological free space" for the multiplication of the tumor cells that normally invade this tissue, or by

releasing, from the irradiated cells of the lymphatic tissue, cell products that stimulate and enhance the growth of the tumor emboli, similar to the stimulating effect of lethally irradiated tumor cells on viable tumor cells, as described by Révész (*32*). It was also shown in our experiments that the immunogenetic conditions of the irradiated chimera influenced, to some extent, the development of these metastases. It has been claimed that the absence of metastases in certain genetic combinations of the irradiated chimera and tumor graft could be attributed to an immunological resistance against the tumor cells in the lymph nodes. It therefore follows that a suppression of the immune mechanism of tumor-bearing animals might stimulate the development of metastases from a nonmetastasizing tumor graft.

It seems relevant, in this connection, to refer to the effect of cortisone on the growth of metastases. It has been shown (*33*, *34*) that tumors will develop metastases if the tumor-bearing animals are injected with cortisone. Cortisone, like total-body X irradiation, was found to elicit the progressive growth of pre-existing metastatic emboli (*34*). It seems possible that the activation of metastases, both by cortisone and X rays, stems from a similar effect of these factors on the resistance capacity of the lymphatic system to tumor metastases. What the nature of this resistance is, whether it involves an immune mechanism (*35*), or whether it resembles a phagocytic mechanism, remains a subject for further analysis.

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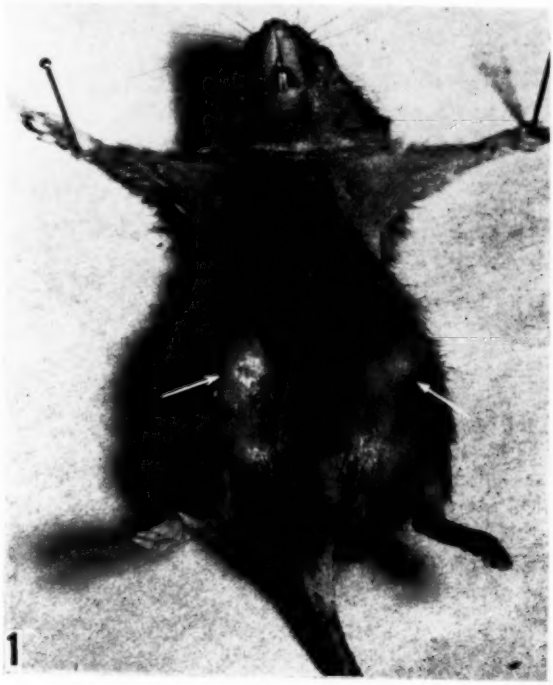
PLATE 13

FIGURES 1 and 2.—Metastases in the inguinal lymph nodes of irradiated C3H mice treated with C3H fetal cells and challenged with a subcutaneous homograft of SBL1 at the dorsal site.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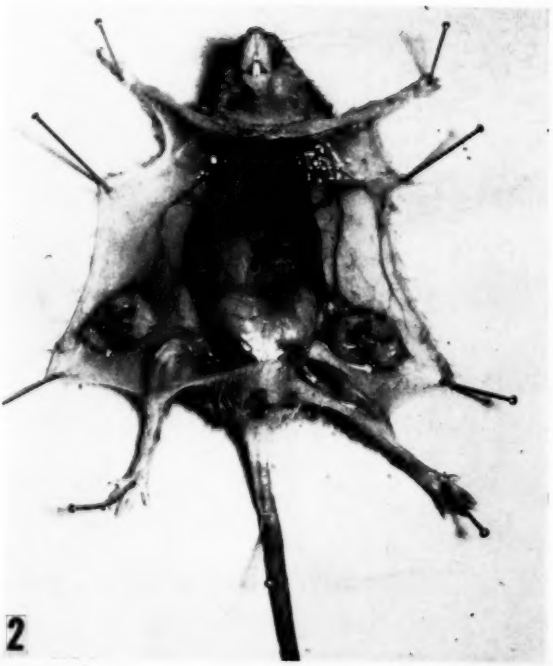
2

Immunogenetic Changes Induced in Tumors Grown in Radiation Chimeras [1,2]

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SUMMARY

An analysis was made of the immunogenetic properties of SBLx tumor lines that had acquired homotransplantability by growing in "radiation chimeras." This study mainly concerned the nature of the induced change and the mechanism of homotransplantability acquired. The SBLx tumors had lost the original antigenicity of SBL1, and the homotransplantability of SBLx was thus based on its inability to elicit an immune response. SBLx homografts, however, were found to be highly susceptible to transplantation immunity. Thus, the low antigenicity of the tumor cells was not due to the loss of the H antigens, but was attributed to a low rate of antigen emission. The analysis of these properties provided evidence that the change of SBL1 to SBLx involved a genetic transformation induced by the conditions that prevailed in the radiation chimeras. The acquired homotransplantability was found to be a reversible process. Transplantation of SBLx tumors back into C57BL or into (C3H × C57BL)F_1 mice resulted in a complete loss of homotransplantability. The mere existence of the isologous C57BL antigens in the host was sufficient to produce this reversed change, and the SBLxB1 tumors thus formed regained the original antigenicity of SBL1. This reversed process took place only if the SBLx tumor was transplanted to hosts of foreign strains following 9 to 16 days of growth in C57BL mice. Prolonged growth of the tumor in C57BL mice did not result in the loss of strain specificity. The significance of these data in relation to the possible mechanism of the reversible change is discussed.—J. Nat. Cancer Inst. 23: 133–151, 1959.

IN A previous paper (*1*) we reported that tumors grafted on X-irradiated mice that were protected by fetal hematopoietic cells may undergo immunogenetic changes manifested in an acquired homotransplantability of the originally strain-specific tumor. The possibility of induced changes in tumor cells following total-body X irradiation has both practical and theoretical implications. The practical aspect stems from the intended application of the principle of "biological protection" to the therapy of leukemia (*2*) and lymphoma-bearing animals (*3*). In these instances, the potential changes that tumor cells remaining after total-body X

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irradiation may undergo is a problem of the utmost importance. The theoretical significance is related to the general problem of directed genetic changes in tumor cells. It has long been known that tumors may acquire homotransplantability after successive transplant generations in hosts of foreign strains, probably through a gradual selection of pre-existing compatible cells (*4–6*). Instances of directed genetic changes that can be induced by a specific experimental treatment are much rarer. The conditions under which a loss of tumor strain specificity may take place, and the immunogenetic properties acquired by the newly homotransplantable tumors, may shed light on the mechanism of the genetic transformation. An analysis of the immunogenetic conditions, in radiation chimeras, that determine such changes was included in our previous investigation (*1*). In the present study, an analysis has been made of the immunogenetic properties of tumors that have acquired homotransplantability by growing in radiation chimeras.

MATERIAL AND METHODS

The C3H and C57BL strains of mice were obtained from the same sources as previously (*1*, *7*). The Swiss mice were of an originally heterogeneous stock that had been bred in our laboratory by brother × sister mating for at least 24 generations.

Tumors.—1) Sarcoma SBL1, produced in C57BL mice (*7*, *8*). This is a strain-specific tumor, which grows only in C57BL mice, and is rejected by all foreign strains tested.

2) SBLx25 and SBLx1, two sublines of Sarcoma SBL1, produced by growing the original SBL1 for one transplant generation in X-irradiated C3H mice inoculated with fetal C3H liver cells (*1*). The tumors were then maintained in normal nonirradiated C3H animals. These are homotransplantable tumors, which give 100 percent successful homografts in a number of foreign strains tested (*1*).

3) The ascites form of 6C3HED lymphoma, produced in C3H mice (*9*), and converted into an ascites form by Klein (*10*). This is a strain-specific tumor, rejected by all foreign strains tested (*11*).

4) The 6C3HED-ICR subline, produced from 6C3HED by its inoculation into fetuses of Swiss ICR (*12*). The original strain-specific tumor was thus adapted to give successful homografts in all foreign strains. The immunological properties of this tumor and the mechanism of its homotransplantability were studied previously (*11*).

5) The ascites form of MC1M and MC1A sarcomas (*10*). Both are of C3H origin; the first is a semihomotransplantable tumor, which gives successful homografts only in the intraperitoneal site, and the second gives successful homografts both by intraperitoneal and intramuscular inoculations (*13*). The immunological properties and the mechanism of homotransplantability of these tumors have been studied previously (*14*).

Antiserums.—C3H anti-C57BL serum was prepared by inoculating C3H mice with cell aggregates of minced C57BL liver and spleen tissue, which were suspended in physiological saline. The cell suspension was inoculated intraperitoneally by trocar. Four to 6 of these inoculations were given at intervals of 7 to 9 days, and the serum was collected about 10 days after the last inoculation. Agglutinins were titrated according to the dextran-human serum method of Gorer and Mikulska (*15*), with 3 percent dextran for antiserum dilution. The erythrocytes tested were suspended in inactivated human serum that had been absorbed with mouse red blood cells and diluted 1:0.5 in saline.

Absorption.—Absorptions of antiserum by tumor cells were made by incubating antiserums diluted, 1 × 4 in saline, with equal volumes of minced tumor tissue. Each sample of antiserum was absorbed twice, each for 1 hour, at 37° C.

EXPERIMENTS

The Mechanism of Homotransplantability of SBLx Sublines

Theoretically, three distinct mechanisms may account for homotransplantability of tumor cells: 1) The capacity of tumors to resist the isoimmune response which their successful homografts elicit while growing progressively in hosts of foreign strains (*14*). 2) The loss of histocompatibility antigens which makes the tumor cells incapable of producing transplantation immunity (*4, 16, 17*). These tumors should, accordingly, be nonsusceptible to the homograft reaction elicited by other cells of the same genetic origin. 3) The low rate of antigen emission which decreases the capacity to immunize hosts of foreign strains. Tumors in this category, although incapable of producing an intense transplantation immunity, should theoretically be susceptible to transplantation immunity produced by other tissue grafts of the same strain, since the tumor cells in these instances contain the histocompatibility antigens. This type of mechanism of successful homotransplantation has not yet been found among tumors. It was shown, however, to apply to the successful homotransplantation of rat ovaries (*18*).

Our experimental approach to determine which category comprises the mechanism of homotransplantation of the SBLx sublines was based on the following assumptions: If the homotransplantability of SBLx is due to its capacity to resist the homograft reaction, and not to its decreased antigenicity, then two *simultaneous* transplantations into the same host of the strain-specific SBL1 and the homotransplantable SBLx should not result in the regression of SBLx, since the latter would resist the homograft reaction produced by SBL1. It is also evident that, if the homotransplantability of SBLx is based on the loss of the histocompatibility antigens, the simultaneous transplantation of SBL1 should not prevent its lethal take, since the immune response produced by SBL1 will not have target

antigens to affect in the SBLx cells. However, if the homotransplantability of SBLx is due to a decreased capacity to immunize, *i.e.*, to emit antigenic material, without a necessary loss in the antigenic content of the tumor cells, then a simultaneous transplantation of SBL1 *should* prevent the growth of SBLx. The following experiments were carried out:

1) One group of C3H mice was challenged with 2 simultaneous transplants of SBLx, one on each hind leg. 2) A second group of C3H mice was similarly grafted, with SBLx on one leg and with SBL1 on the other. 3) A third group of C3H was similarly grafted, with SBLx on one leg and with C57BL spleen cells on the other. 4) A fourth group was grafted, with SBLx on one leg and, on the other, with a piece of striated muscle from the leg of a C57BL mouse. The results (table 1) were that, although the SBLx did not elicit an immune response intense enough to prevent its own growth, this tumor was very susceptible to transplantation immunity produced by other C57BL cells. Mice grafted with SBLx on 2 legs showed 5 of 5 lethal takes of the tumor, whereas all the animals grafted, on one leg, with SBL1 and with SBLx on the other have rejected the homotransplantable SBLx. Similar results were obtained when one leg was grafted with SBLx and the other with C57BL spleen cells. The SBLx was rejected due to the immune response produced by the C57BL spleen cell. However, the simultaneous transplants of muscle cells on one leg and of SBLx on the other did not prevent the lethal take of SBLx.

TABLE 1.—Susceptibility of SBLx homografts to the immune response produced by simultaneous grafts of SBL1 or normal C57BL tissues

Group No.	Hind leg inoculated	Tumor grafted	Lethal takes of tumors
1	Right	SBLx	5/5
	Left	SBLx	5/5
2	Right	SBL1	0/5
	Left	SBLx	0/5
3	Right	C57BL spleen	—
	Left	SBLx	0/5
4	Right	C57BL muscle	—
	Left	SBLx	5/5

These results show that the level of transplant immunity produced by SBL1 or by C57BL spleen cells was higher than that produced by SBLx. The progressive growth of SBLx, when grafted simultaneously with C57BL muscle cells, should be attributed to the low antigenicity of muscle cells. It has been shown that the isohemagglutinogen content in muscles is lower than in other tissues (*19*). It seems probable that the transplantation antigens, which are determined by the same gene complex that determines the hemagglutinogens, are also weaker in muscles than in other somatic tissues such as liver or spleen.

Thus, the homotransplantability of SBLx sublines is based on a decreased antigenicity of this tumor and not on an increased resistance to

transplantation immunity. The susceptibility of SBLx homografts to the immune response evoked by other tissue grafts indicates that the decreased antigenicity of SBLx *is not due to a loss of the histocompatibility antigens in the tumor cells.*

The mechanism of homotransplantability of SBLx differed from that of "resistant tumors." Further evidence for the distinction between these two mechanisms of homotransplantability was provided by subjecting the "resistant tumors" to simultaneous transplantation tests similar to those performed with SBLx.

It was previously shown that the tumors MC1M, MC1A, and 6C3HED-ICR, which originated in C3H mice, produced an intense immune response while growing progressively in foreign strains, and therefore their homotransplantability was attributed to their capacity to resist the homograft reaction they themselves produced (*11*, *14*). In this instance, simultaneous homografts of the strain-specific tumors of the same genetic origin should *not* prevent the progressive growth of MC1A and 6C3HED-ICR. The following experiments were carried out (table 2):

TABLE 2.—Progressive growth of homografts of MC1A and 6C3HED-ICR inoculated simultaneously with 6C3HED and MC1M tumors

Group No.	Hind leg inoculated	Tumor grafted	Lethal takes of tumors
1	Right	6C3HED-ICR	5/5
	Left	6C3HED-ICR	5/5
2	Right	6C3HED	0/5
	Left	6C3HED-ICR	5/5
3	Right	6C3HED	0/5
	Left	MC1A	5/5
4	Right	MC1A	6/10
	Left	MC1A	6/10
5	Right	MC1M	0/15
	Left	MC1A	12/15
6	Right	MC1A	7/9

6C3HED-ICR.—1) Each of a group of 5 Swiss mice received 2 simultaneous inoculations of 6C3HED-ICR. 2) A second group was similarly inoculated with 6C3HED-ICR on one leg and, on the other, with the strain-specific 6C3HED. The results were that the animals challenged with 2 inoculations of 6C3HED-ICR gave successful homografts on both legs (table 2, group 1), and those inoculated with 6C3HED on one leg and, on the other, with 6C3HED-ICR gave a regression of the strain-specific tumor but a lethal take of the 6C3HED-ICR (table 2, group 2).

MC1A.—1) One group of 10 Swiss mice was challenged with 2 simultaneous inoculations of MC1A. 2) A second group of 5 Swiss mice was challenged with 6C3HED on one leg and with MC1A on the other. 3) A third group of 15 Swiss mice was challenged with MC1M on one leg and with MC1A on the other. The results were that simultaneous transplants

of the regressing 6C3HED (table 2, group 3), or of the regressing MC1M (group 5), did not cause a regression of the homotransplantable MC1A. Five of 5 MC1A homografts inoculated with 6C3HED and 12 of 15 MC1A inoculated with MC1M gave successful homografts. The control group of 2 inoculations of MC1A (group 4) gave 6 of 10 lethal takes. In this instance, the growth of the MC1A in the experimental groups was even more pronounced than in the control animals. Another test was made in which 5 Swiss mice were given, simultaneously, 1 intraperitoneal inoculation of 6C3HED and 2 intramuscular inoculations of MC1A. Here, also, the regressing 6C3HED did not cause a rejection of the MC1A; 4 of 5 animals gave lethal takes of the 2 intramuscular MC1A inoculations (table 3, group 4).

TABLE 3.—Progressive growth of homografts of MC1M and MC1A inoculated simultaneously with 6C3HED and MC1M tumors

Group No.	Site of inoculation*	Tumors grafted	Lethal takes of tumors
1	I.P.	MC1M	5/5
	I.M.	6C3HED	0/5
2	I.P.	MC1M	5/5
3	I.M.	MC1M	0/5
	I.P.	MC1M	5/5
	I.P.	6C3HED	0/5
4	I.M. right	MC1A	4/5
	I.M. left	MC1A	4/5

*I.P. = Intraperitoneal inoculation.
I.M. = Intramuscular graft.

MC1M.—The MC1M ascites sarcoma is homotransplantable only when injected intraperitoneally. Therefore, the experiment with this tumor was performed by challenging Swiss mice with an intraperitoneal inoculation of MC1M and a simultaneous intramuscular inoculation of either MC1M or 6C3HED. One group of 5 Swiss animals was injected intraperitoneally with MC1M and intramuscularly with MC1M. A second group of 5 Swiss animals was injected intraperitoneally with MC1M and intramuscularly with 6C3HED. In both groups the intramuscular grafts were rejected, but all the animals died of progressively growing intraperitoneal tumors (table 3).

It can be concluded that the homotransplantable C3H tumors MC1M, MC1A, and 6C3HED-ICR have shown complete resistance to the homograft reaction produced by simultaneous transplants of the strain-specific tumors, in contradistinction to the high susceptibility of SBLx to a similar reaction.

The Antigenicity of SBLx

The experiments with simultaneous tumor grafts have shown that the SBLx was very susceptible to the immune response produced by SBL1, and consequently the conclusion was drawn that SBL1 produced a more

TABLE 4.—Immunizing effect of subcutaneous grafts of SBL1 and SBLx tumors* on subsequent intramuscular grafts of SBLx†

First tumor graft	Second tumor graft	Lethal takes of second graft
SBL1	SBLx	1/15
SBLx	SBLx	10/15

*Removed after 7 days of growth.
†Transplanted 9 days after the transplantation of the first graft.

intense immune response than SBLx. In fact, the results of those experiments could be interpreted differently in relation to the last conclusion. One may assume that the SBLx homograft does elicit transplantation immunity similar to SBL1, but that the immune response of SBL1 is produced earlier. In simultaneous tumor grafts, the "early" homograft reaction produced by the SBL1 attacks the SBLx in its susceptible stage, *i.e.*, before the tumor becomes established in the foreign host, and therefore causes its regression. Accordingly, the level of the "late" immune response produced by SBLx need not be lower than that produced by SBL1; its homotransplantability will be based on its capacity to resist this late reaction.

To test this antigenicity, 2 groups of C3H animals were challenged, one with a subcutaneous SBL1 graft and the other with a subcutaneous SBLx graft. After 7 days of initial growth, all the tumors were excised under sodium pentobarbital (Nembutal®) anesthesia, and 2 days later the animals of both groups were each challenged with an intramuscular graft of SBLx. It was obviously expected that a first graft of SBL1 would inhibit the progressive growth of a second SBLx graft. But, on the assumption that SBLx does produce a "late" immune response, and in view of its high susceptibility to transplantation immunity, one would also expect a first graft of SBLx to inhibit the progressive growth of a second SBLx, which was grafted 9 days after the first one.

It was found (table 4) that the second SBLx graft was rejected by animals that were preimmunized with SBL1, but among those that were first challenged with SBLx, 10 of 15 gave lethal takes of the second SBLx graft. These results indicate that, although SBLx homografts can elicit transplantation immunity, its intensity is comparatively very low: One graft of SBL1 immunized 14 of 15 C3H mice against a second SBLx graft, whereas only 5 of 15 animals were similarly immunized by a first graft of SBLx. It can now be definitely concluded that the homotransplantability of SBLx is not based on its capacity to resist the homograft reaction but is due to the decreased antigenicity of this tumor.

The Reversibility of the Acquired Homotransplantability of Sarcoma SBLx

In our previous study (*1*) we have shown that the transformation toward homotransplantability of SBL1 was most intense when the tumor

was grafted into irradiated C3H mice inoculated with C3H fetal hematopoietic cells. It was also found that if other experimental systems of irradiated hosts were used, the greater the contribution of C57BL antigens (*i.e.*, tissue isologous to the tumor) in the irradiated recipient, the lesser the change in the strain specificity of SBL1. It appeared that the existence of C57BL antigens in the irradiated animal tended to inhibit the development of homotransplantability. It seemed of interest, therefore, to test whether the homotransplantability of the transformed tumor (SBLx) could be similarly inhibited, or reversed, by regrafting the SBLx into isologous nonirradiated C57BL or (C3H $\times$ C57BL)F_1 recipients. The following experiments were carried out:

SBLx tumors that had been transferred for 7 to 11 generations in C3H mice (and gave 100% lethal takes in these animals) were grafted back to C57BL mice. At various intervals following their transplantation to the C57BL recipients, the tumors were excised, the host's capsular connective tissue was completely removed, and then the tumors were regrafted intramuscularly into C3H mice and tested for growth. In the first experiment (table 5), tumors of the subline SBLx25 were used. They were transplanted intramuscularly and subcutaneously to C57BL mice and, at 9, 10, 11, 12, 16, 20, 22, and 25 days of growth, the animals were killed and the tumors transplanted back into C3H hosts. The tumor from each C57BL donor was implanted into 4 to 8 recipients. The results (table 5) were that tumor grafts, which had been growing for 9 to 16 days in C57BL, were *all* rejected by the C3H animals. The regression of the tumors in the C3H hosts took place within 7 to 20 days following transplantation. It thus appeared that the homotransplantable SBLx tumor, by regrafting into its isologous C57BL mice, had regained its strain specificity.

An unexpected result has been found, however, with tumors that had grown in C57BL hosts for more than 20 days. These have *not* shown a similar loss of the acquired homotransplantability (table 5). One tumor that had grown for 20 days in a C57BL mouse gave 3 of 8 lethal takes when it was regrafted back to C3H. A second tumor that was transferred after 22 days of growth gave 5 of 6 lethal takes, and a third tumor that was transplanted after 25 days of growth resulted in 4 of 4 lethal takes in C3H mice. It seems, therefore, that the growth of SBLx in the isologous mice for 9 to 16 days was accompanied by the loss of homotransplantability. A prolonged growth for 25 days was not accompanied by such a loss.

The apparently contradictory results of the loss of homotransplantability, if the tumor grows for 9 to 16 days in C57BL hosts, and of the retention of homotransplantability, if the tumor grows for more than 22 days, suggest that the SBLx evokes a reaction in the C57BL host that is responsible for the loss of homotransplantability. It appears that this loss takes place only if the SBLx is grafted back to the foreign C3H mice during the acting period of the host's reaction, *i.e.*, during the 9th to 16th day. To test this assumption, an experiment was performed in which SBLx tumors were grafted into C57BL mice. After 12 days of growth,

TABLE 5.—Effect of growth of SBLx25 in C57BL mice on loss of acquired homotransplantability

Site of growth in C57BL mice*	Period of growth in C57BL mice (*days*)	Growth of SBLxB1 in C3H mice prior to regression (*days*)	Lethal takes of SBLxB1 in C3H mice
I.M.	9	<12	0/6
S.C.	9	<12	0/6
I.M.	10	<7	0/8
I.M.	11	<12	0/6
S.C.	11	<7	0/5
I.M.	12	<20	0/5
I.M.	16	<12	0/6
S.C.	16	<12	0/6
S.C.	16	<7	0/5
I.M.	20	<16 (5/8)	3/8
S.C.	22	<15 (1/6)	5/6
I.M.	25	—	4/4
I.M. × 2†	12 + 12	<16	0/5

*I.M. = Intramuscular inoculation.
S.C. = Subcutaneous inoculation.
†*See* text.

the tumors were grafted into other C57BL mice and, after another 12 days in these secondary hosts, they were transplanted back to C3H mice. It was assumed that if a response of the C57BL hosts, which functioned around the 12th day, determined the loss of homotransplantability, then, although in this experiment the tumors grew in C57BL mice for a total period of 24 days, they should still show a loss of homotransplantability, since they were grafted to C3H mice on their 12th day of growth in the secondary C57BL hosts. The results (table 5, *bottom line*) confirm this assumption. All tumors grafted to C3H animals regressed within 16 days. It is thus indicated that there is a critical period during the growth of SBLx in its isologous host at which the loss of homotransplantability is induced, and this suggests the function of a host's response. These experiments did not indicate, however, the nature of this host's postulated reaction.

The strain specificity regained by retransplanting SBLx into C57BL mice was checked with another subline of SBLx, namely, SBLx1. Tumors of this subline were grafted intramuscularly and subcutaneously into C57BL mice, and 11 and 16 days later they were regrafted back to C3H mice. The results (table 6) were that the tumors were always rejected by the C3H hosts within 9 days after transplantation. This tumor had thus also gained its original strain specificity by growth in its original isologous environment.

To test whether the pure isologous C57BL host is required to induce the loss of homotransplantability, *i.e.*, whether the *lack* of foreign antigens is the determining factor, or whether the mere existence of the isologous C57BL antigens is sufficient for the induction of this change, the SBLx

TABLE 6.—Effect of growth of SBLx1 in C57BL mice on loss of acquired homotransplantability

Site of growth in C57BL mice	Period of growth in C57BL mice	Growth of SBLxB1 in C3H mice prior to regression (*days*)	Lethal takes of SBLxB1 in C3H mice
I.M.	11	≤ 7	0/5
S.C.	11	≤ 7	0/5
I.M.	16	≤ 9	0/5
S.C.	16	≤ 9	0/5

tumor was grown in mice containing both C57BL and C3H antigens, and then was tested for homotransplantability.

SBLx25 tumors were transplanted to (C3H × C57BL)F_1 mice. After 10 and 20 days of growth, the tumors were excised and transplanted back to C3H animals. The results (table 7) were that here, too, a loss of homotransplantability was obtained. Thus, the mere existence of C57BL antigens was sufficient to produce the loss of homotransplantability.

In our previous experiment (table 1) we found that the original strain-specific SBL1, if grafted intramuscularly into C3H mice with a simultaneous homograft of SBLx, caused a regression of the homotransplantable SBLx. The SBL1 tumor was thus shown to elicit a more intense homograft reaction than that produced by the SBLx. To test whether the SBLx, which gained its strain specificity by its growth in C57BL hosts, namely, the SBLxB1, had, in fact, acquired the high antigenicity that characterizes the SBL1 tumor, SBLxB1 was subjected to "simultaneous transplantation tests" similar to those described previously but in which the SBLxB1 was the "immunizing graft" instead of the SBL1, as in the previous experiments. To find out whether the SBLxB1 would behave similarly to SBL1 regarding its capacity to prevent the progressive growth of a simultaneous homograft of SBLx, the following experiment was carried out: 1) One group of 5 C3H mice was grafted with SBLx on one leg, and, on the other, with SBLxB1, which had grown for 13 days in C57BL mice. 2) A second group of 5 C3H mice was grafted with SBLx on one leg, and, on the other, with SBLxB1, which had grown for 25 days in C57BL mice. 3) A third control group of 5 C3H mice was grafted, on both legs, with SBLxB1, which had grown for 13 days in

TABLE 7.—Effect of growth of SBLx25 in (C3H × C57BL)F_1 mice on loss of acquired homotransplantability

Site of growth in (C3H × C57BL)F_1	Period of growth in F_1 mice (*days*)	Growth of SBLxB1 in C3H mice prior to regression (*days*)	Lethal takes of SBLxB1 in C3H mice
I.M.	10	>8	0/9
I.M.	20	>14 (7/8)	1/8

C57BL mice. The results (table 8) were that the SBLxB1 taken from C57BL hosts after 13 days of growth had virtually inhibited the otherwise lethal take of SBLx in C3H mice: Only 1 of 5 animals developed successful homografts of the 2 tumors. In this respect, the SBLxB1 behaved similarly to the SBL1 tumor. However, SBLxB1, after 25 days of growth in C57BL, which, as shown previously (table 5), had not lost its homotransplantability, did not prevent the progressive growth of the simultaneous homograft of SBLx: 5 of 5 animals showed lethal takes of the 2 tumors. The control group of SBLxB1, after 13 days of growth in C57BL grafted on 2 legs of C3H, showed, as expected, a complete regression of the tumor.

TABLE 8.—Effect of SBLxB1 homograft on the fate of a simultaneous SBLx graft in C3H mice

Group No.	Hind leg inoculated	Tumor grafted	Growth period of SBLxB1 in C57BL prior to inoculation (*days*)	Lethal takes of tumor
1	Right	SBLxB1	13	1/5
	Left	SBLx		1/5
2	Right	SBLxB1	25	5/5
	Left	SBLx		5/5
3	Right	SBLxB1	13	0/5
	Left	SBLxB1	12	0/5

The Serological Properties of SBL1 and SBLx Tumors

From two viewpoints attempts were made to study the serological properties of SBL1 and SBLx tumors: 1) the antigenicity of the tumors, as reflected in the production of isohemagglutinins by homografts of the 2 tumors, and 2) the distribution of C57BL agglutinogens in SBL1 and SBLx cells, as demonstrated by the absorptive capacity of the tumor cells. Our approach to the study of the antigenicity of the tumors proved completely disappointing, since SBL1 and SBLx homografts in C3H mice produced no hemagglutinins. C3H mice were challenged with subcutaneous homografts of either SBL1 or SBLx. The animals were tumorectomized after 7 days (to prevent absorption *in vivo* of agglutinins by the tumor cells) and tested for hemagglutinins, between the 10th and 16th day after tumor transplantation, against C57BL red blood cells. No antibodies were found. The tumorectomized animals were then regrafted with 3 successive tumor transplants. But even 4 such grafts failed to yield any detectable amount of antibodies. The anti-SBLx serum was completely negative like the anti-SBL1 serum, in all but one instance in which traces of agglutinins with a titer of 1×16 were found.

Absorptions.—Anti-C57BL serum was produced in C3H mice by successive intraperitoneal inoculations of C57BL spleen and liver tissues; the antiserum for absorptions was of a titer of $>2^{10}$.

The first test was made in order to compare the absorptive capacities of SBL1 and SBLx tumors. Anti-C57BL serum was divided into 4 samples. One sample was absorbed with a SBLx tumor (of the subline SBLx25) grown in C3H mice, the second with SBLx grown in C57BL mice (*i.e.*, SBLxB1), the third with SBL1 grown in C57BL mice, and the fourth sample was left unabsorbed. The tumor cells were all from subcutaneous grafts, excised for absorption on the 18th day of growth. Each sample was absorbed twice, at 37° C., and then tested for agglutinins against C57BL erythrocytes. Table 9 shows that the SBL1 cells carried out complete absorption of anti-C57BL agglutinins. A similar complete absorption was obtained with SBLxB1, *i.e.*, with SBLx grown in C57BL. On the other hand, SBLx from C3H hosts did *not* remove the anti-C57BL agglutinins. It thus appears that SBLxB1 is similar in this respect to SBL1, whereas SBLx behaves in a completely different manner. This corresponds to the similarity in strain specificity and in the capacity to elicit transplant immunity found in these 2 tumors in our previous experiments. These results were repeatedly obtained with tumors at 9, 19, 23, and 27 days of growth in C3H recipients, and with SBLx of the 2 sublines SBLx25 and SBLx1.

The striking differences in the absorptive properties between SBLx and SBLxB1 and SBL1 can be explained by either of the following assumptions:

1) The transformation of SBL1 to SBLx involved the loss of one or more of the C57BL isoagglutinogens. This would explain the lack of absorption by SBLx. If the reversed transformation in the homotransplantability properties produced by the growth of SBLx in C57BL involved the reappearance of the lost agglutinogens, one would obviously expect the SBLxB1 to perform a complete absorption of anti-C57BL like SBL1; in fact, these were the results obtained.

2) No agglutinogens had been lost by the transformation of SBLx. On the contrary, while growing in C3H mice, the SBLx cells became sensitized with anti-C57BL agglutinins which the cells themselves elicited. Hence, having absorbed the anti-C57BL agglutinins *in vivo*, the SBLx cells from C3H mice were saturated with antibodies and could not, therefore, absorb agglutinins *in vitro*.

To test the feasibility of the sensitization theory as an explanation for the lack of absorption by SBLx, a further experiment was carried out in which SBLx cells for absorption were taken from the subcutaneous tumor of an 8-day-old homograft. Assuming that the lack of absorption by SBLx is due to its saturation with antibodies elicited by the tumor, we expected that, following 8 days of growth in C3H hosts, a graft would still be uncoated with agglutinins; if that occurred then the SBLx should perform a complete, or at least a more intense, absorption than SBLx from older grafts. The results (table 10) were, in fact, that there were no differences between the absorption of SBLx at 8 and at 18 to 27 days of growth. The 8-day-old cells removed practically no anti-C57BL agglutinins. These observations, although they are by no means proof, tend to indicate

TABLE 9.—Absorption of C3H anti-C57BL serum by SBL1, SBLx, and SBLxB1 cells*

Antiserum absorbed with tumor cells of:	Absorbed antiserum diluted 1:									
	8	16	32	64	128	256	512	1024	2048	4096
SBL1	−	−	−	−	−	−	−	−	−	−
SBLx	+++	+++	+++	+++	+++	+++	++	+	±	−
SBLxB1	−	−	−	−	−	−	−	−	−	−
Unabsorbed	+++	+++	+++	+++	+++	+++	+++	+++	++	++
Control normal serum†	−	−	−	−	−	−	−	−	−	−

*Antiserum tested against C57BL red blood cells.
†Normal C3H serum tested against C57BL red blood cells.

TABLE 10.—Absorption of C3H anti-C57BL serum by cells of SBLx from 8-, 18-, and 27-day-old grafts

Antiserum absorbed with tumor cells of:	Age of tumor cells (*days*)	Absorbed antiserum diluted 1:									
		8	16	32	64	128	256	512	1024	2048	4096
SBLx	8	+++	+++	+++	+++	+++	+++	+	+	±	−
SBLx	18	+++	+++	+++	+++	++	++	±	−	−	−
SBLx	27	+++	+++	+++	+++	++	+	±	−	−	−
SBL1	23	−	−	−	−	−	−	−	−	−	−
Unabsorbed serum		+++	+++	+++	+++	+++	+++	+++	+++	+	+
Control normal C3H serum		−	−	−	−	−	−	−	−	−	−

TABLE 11.—Absorption of anti-BALB/c agglutinins from C3H anti-C57BL serum

C3H anti-C57BL serum absorbed with tumor cells of:	Tested against RBC of:	Absorbed antiserum diluted 1: 8	16	32	64	128	256	512	1024	2048	4096
Unabsorbed	C57BL	+++	+++	+++	+++	++	++	++	+	±	−
Unabsorbed	BALB/c	+++	+++	+++	+++	++	++	++	+	±	−
SBL1	BALB/c	−	−	−	−	−	−	−	−	−	−
SBLx	BALB/c	+++	+++	++	++	+	+	+	±	−	−
Control normal C3H	BALB/c	−	−	−	−	−	−	−	−	−	−

that the lack of absorption by SBLx is not due to the fact that the cells are coated with agglutinins. An indirect support for this conclusion may also be that SBLx produces little, if any, agglutinins. It is hard to conceive that a tumor, which fails to elicit detectable agglutinins even after 4 transplants into one host, can still produce them in a sufficient amount to cause complete saturation of the tumor cells of the first homograft. A critical experiment, to clarify this point further, was carried out by the absorption of antiserums with SBL1 taken from C3H hosts at 8 days of growth. If no saturation occurs at this age, then, unlike SBLx, the SBL1 grown in C3H hosts *should* carry out a complete absorption. However, since SBL1 regresses rather rapidly in C3H hosts, this experiment was complicated and the results are still unclear. The tumor sometimes removed all, and in other cases only part, of the antibodies. Further experiments are under way. If it can be shown that SBL1 from C3H hosts can absorb anti-C57BL, and SBLx from C3H hosts cannot, the sensitization theory will certainly lose much of its basis.

An attempt was made to test the idea of a loss of antigens, by postulating that if only certain types of antigens of the *H-2* complex of C57BL strain (D^bEF) are lost by SBLx, such as the D^b antigen, and that if this tumor still retains the F antigen, which is also shared by the BALB/c, then C3H anti-C57BL serum, absorbed by SBLx, should remove the anti-BALB/c agglutinin (F) from this antiserum. Such a result would support the idea of a partial loss in the antigenic complex of SBLx. C3H anti-C57BL serum was therefore absorbed with SBLx and tested against BALB/c red blood cells. Table 11 shows that the SBLx cells did *not* absorb the anti-BALB/c agglutinins. In C3H anti-C57BL serum, no anti-E can be formed, since the C3H itself contains the E antigen, but there still remained the possibility that SBLx has lost the D^b antigen.

No definite conclusion can yet be drawn to explain the differences in the absorptive properties between SBLx and SBL1. It does not, however, seem to us that the sensitization *in vivo* in C3H hosts is the answer to this question, but so far we have no clear evidence for the idea of loss of antigens. The similarity in the absorptive properties of SBL1 and SBLxB1, as distinct from those of SBLx, is in striking harmony with the other imunogenetic properties of these tumors, but its basis awaits further analysis.

DISCUSSION

Transplantation experiments with SBLx sublines have provided evidence that the homotransplantability of these adapted tumors was based on their inability to elicit immunity on a functional level. In spite of the high susceptibility of the adapted tumors to the immune response evoked by C57BL antigens of other cell types, such as the SBL1 or C57BL spleen cells, the SBLx tumor itself could produce only a very weak homograft reaction against a subsequent transplant of SBLx. This inability

to produce transplantation immunity could therefore be attributed to a low rate of emission of antigens by the SBLx cells, *i.e.*, to a decreased antigenicity of the adapted tumor, and this would then account for the progressive growth of its homografts. The low antigenicity does not, however, reflect an actual loss of transplantation antigens. It is clear that the SBLx cells not only possess the original H antigens, but that these seem to be vital in the tumor cells, as shown by the high susceptibility of SBLx to the immune response produced by a simultaneous SBL1 homograft. Had the antigenicity of SBLx been determined by the absence of C57BL histocompatibility antigens, one obviously would not expect the SBLx cells to be so highly susceptible to transplantation immunity.

In this respect the SBLx tumor differs fundamentally from the "resistant tumors," such as the homotransplantable C3H-originated MC1M and MC1A sarcomas (*14*). One intraperitoneal MC1A homograft, excised after 7 days, produced an immune response high enough to prevent the progressive growth of a subsequent MC1A. An intraperitoneal inoculation of MC1M similarly prevented the growth of a second intraperitoneal homograft of the same tumor. However, one SBLx homograft failed to inhibit the progressive growth of a subsequent graft given 9 days later. The regression of the second MC1M and MC1A homografts should *not* be attributed to a high susceptibility of these tumors to transplantation immunity. On the contrary, these tumors were shown to resist high levels of transplantation immunity. The MC1A tumor grew progressively, not only when inoculated intramuscularly with a simultaneous 6C3HED homograft, as shown in the present investigation, but also when inoculated intraperitoneally 7 days subsequent to a preceding intraperitoneal inoculation of the strain-specific 6C3HED (unpublished data). MC1A gave a successful homograft when inoculated after a first intraperitoneal MC1M graft (*14*). Similar results were obtained with 6C3HED-ICR and DBA-ICR inoculated subsequent to homografts of their strain-specific sublines (*11*). These data indicate that the homotransplantable MC1A produces a stronger immune response than the strain-specific 6C3HED, or the semi-homotransplantable MC1M. The SBLx, however, produces a much weaker immune response than the strain-specific SBL1. The growth of MC1A and 6C3HED-ICR in foreign hosts is attributed to their capacity to resist the immune response they themselves produce. The growth of SBLx in foreign hosts is attributed to the fact that they do not produce transplantation immunity.

These immunogenetic properties of SBLx indicate that the mechanism of change from SBL1 to SBLx is not based on a gradual selection of immunologically compatible cells from the original heterogeneous cell populations of SBL1. A selective pressure exerted by a low immune response formed by the original SBL1 cells, which, theoretically, could have been expected in radiation chimeras, would have selected only those cells that are *unsusceptible* to transplantation immunity due to a loss of H antigens. Under conditions of immune pressure, there is obviously no selective

advantage to cells that are highly susceptible to transplantation immunity, as the SBLx cells are. It therefore seems inconceivable that the SBLx tumor was adapted by a selective mechanism; it seems much more probable that the SBLx tumors were produced by an induced transformation in the original tumor cells. This conclusion is in accord with that derived from the analysis of the genetic factors in the radiation chimeras involved in the adaptation of SBLx (*1*).

If the low antigen emission represents the basis of homotransplantability of the SBLx tumor, it follows that the genetic change reflected in the loss of strain specificity was not fundamentally a loss of H factors (although this might have also occurred concurrently), but rather a change in the genetic control of the metabolic pathways of the histocompatibility antigens. A lower rate of synthesis of transplantation antigens, an increased rate of degradation, or a lower rate of extracellular emission of antigens might explain the low antigenicity of the SBLx tumor. Each of these assumed processes must be genetically determined. However, without any evidence as to the chemical nature of the transplantation antigens, this postulated metabolic basis for the low antigenicity cannot yet be subjected to an experimental test.

Repeatedly, in our experiments, SBLx, when regrafted into C57BL mice, regains its strain specificity. This reversed change is determined by the mere existence of the isologous C57BL antigens, since the transplantation of SBLx into (C3H $\times$ C57BL)F_1 mice results also in the loss of homotransplantability. The reversed tumor (SBLxB1) has gained its original antigenicity, as demonstrated by its ability to prevent the progressive growth of a simultaneous SBLx homograft. This regaining of strain specificity by *one passage* through hosts of the tumor strain of origin, or F_1 hybrid hosts, is, in fact, a unique phenomenon. Some analogous modifications toward an increased specificity have been described previously, but these have been achieved only after serial passages in mice of the tumor's strain of origin. Strong has reported (*20*) that a PBR spindle-cell sarcoma that had been transferred in mice of N strain for 9 transplant generations has been returned to PBR animals and transplanted there for 13 generations. The transplantability of the tumor was then tested in (PBR $\times$ N)F_2 animals, and it gave a lower percentage of takes than the original tumor. In this instance the increased specificity of the tumor was not a result of a reversed adaptation, since the specificity that was conferred upon it was greater than that of the original tumor. Molomut (*21*) has described experiments with Sarcoma I (SaI) of strain A origin, which has acquired homotransplantability by growing in C57BL mice treated with lyophilized SaI tissue. Two sublines of the adapted SaIMo were grafted back to mice of strain A, and as a result one subline regained its strain specificity after 11 passages in strain A mice, and the other after 20 passages. It seems that in this instance the strain-specific cells were *gradually* selected by the strain A mice.

The regaining of strain specificity by the SBLx tumor was achieved after 9 to 16 days of growth in C57BL animals. If the tumor grows for a longer

period in C57BL hosts and is then tested in foreign strains, homotransplantability is retained. The existence of a crucial period during the growth of the tumor in C57BL, in which the loss of homotransplantability is induced, suggests the possibility that a host's reaction is involved in the reversed change. The very fact that this change takes place between the 9th and 16th day after transplantation points to a host's response similar to that of the homograft reaction. Had the adaptation of SBLx in the radiation chimera involved the gain of some new antigenic components, which were important in the acquired homotransplantability, then the idea of a homograft reaction directed against the postulated antigen(s), which caused its inactivation, might have been a possible explanation. However, since the reversed change can occur in (C3H × C57BL)F_1 animals that are not theoretically active immunologically against the tumor, that is, in any environment *in vivo* containing the C57BL antigens, and does not occur in the absence of C57BL antigens, it seems more likely that the C57BL antigens *per se* are the active factor in the induction of strain specificity, and not an immune response produced by C57BL cells. How these antigens exert their influence remains a subject for further investigation.

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5-Bis (2-Chloroethyl) Aminouracil, a New Antitumor Agent [1, 2]

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SUMMARY

5-Bis(2-chloroethyl)aminouracil U-8344 has been synthesized and found to be an active oncolytic agent against the Walker carcinosarcoma 256 in rats. The compound, when administered to these tumor-bearing rats in 3 to 5 doses of 2.0 to 4.0 mg. per kg. orally at intervals of 2 to 8 days, has the capacity to cause complete regression of established tumors. Many of the usual side effects of nitrogen mustards are absent from the action of this drug. The drug is more efficacious when given intermittently than when given daily.—J. Nat. Cancer Inst. 23: 153-162, 1959.

THE BIOLOGICAL and pharmacological properties of methyl-bis(2-chloroethyl)amine were first described by Gilman and Philips (*1*) and led to the use of this compound in the treatment of Hodgkin's disease and lymphoma in man. Since then, many investigators have studied the problem of improving the oncotoxicity of the nitrogen mustard grouping and at the same time eliminating many of its serious side effects. These studies have resulted in the availability of a number of new nitrogen mustard derivatives which have clinical value, as is indicated by the recent review of Farber *et al.* (*2*).

Despite the advances which have been made in this field, the usefulness of nitrogen mustards and of alkylating agents in general has been limited, especially in regard to their efficacy against solid tumors. They also lack specificity of antitumor activity and possess high general toxicity. Our interest in the field of cancer chemotherapy and particularly in the oncolytic activity of the nitrogen mustard grouping has led to the synthesis of 5-bis(2-chloroethyl)aminouracil (*3*), which we have found to be a very active antitumor agent with fewer side effects than those usually encountered with nitrogen mustards.

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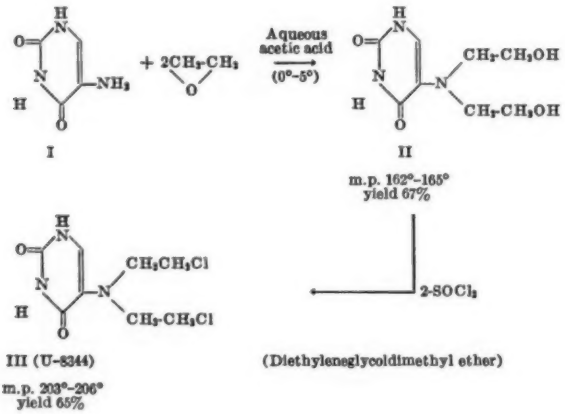
EXPERIMENTS

The synthesis of 5-bis(2-chloroethyl)aminouracil was accomplished according to the method outlined in text-figure 1.



TEXT-FIGURE 1.

5-Bis(2-hydroxyethyl)aminouracil (II)

Fifty gm. of 5-aminouracil (0.393 mole), 875 ml. of water, and 55 ml. of glacial acetic acid were placed in a 2-l. flask, and the mixture was chilled in an ice bath, with stirring. To the cold (0–5° C.), stirred mixture was added 300 ml. of ethylene oxide. The temperature was maintained at 0 to 5° C. for 2 hours, then allowed to come slowly to room temperature. Stirring was continued at room temperature for 2 days. After the solid was all dissolved, the flask was evacuated to remove most of the excess ethylene oxide. Seven hundred ml. of water was added, followed by 200 gm. of wet Dowex-50 resin (H+ form). The mixture was stirred for 2 hours, then the resin was collected on a filter and washed with 3 × 200 ml. of water. The wet resin was stirred with 450 ml. of 10 percent ammonium hydroxide for 1 hour to elute the product. The resin was removed by filtration and washed with 200 ml. of 5 percent ammonium hydroxide, then with 150 ml. of water. The filtrate and washes were combined and the solvent was evaporated under vacuum, leaving a white, gummy solid. This solid was heated with 700 ml. of isopropyl alcohol. The material which remained undissolved was removed by filtration and washed with a little hot isopropyl alcohol. The filtrate was concentrated at atmospheric pressure until crystallization began. It was chilled at 4° C. overnight. The crystals were collected, washed with cold isopropyl alcohol, and dried under vacuum; weight, 56.7 gm. (67%), m.p. 162 to 165° C. (block). This material was found to be suitable for use in the next step.

An analytical sample was prepared by recrystallizing this material from isopropyl alcohol; m.p. 166 to 168° C.

Analysis calculated for $C_8H_{13}N_3O_4$: C, 44.64; H, 6.09; N, 19.53. Found: C, 44.60; H, 6.55; N, 19.19.

The ultraviolet absorption spectrum of (II) in 0.01 N H_2SO_4 in 95 percent ethanol indicated the following maxima: 258 mμ–aM 5,600; 204 mμ–aM 8,250.

5-Bis(2-chloroethyl)aminouracil (III) U–8344

Ethylene glycol dimethyl ether (310 ml.), 8.0 ml. of ethyl alcohol, and 14 drops of water were placed in the 500-ml. flask and stirring was started. Thionyl chloride, 11 ml., was added carefully from the dropping funnel. When the reaction was over, the flask was cooled in water at 10° C. 5-Bis(2-hydroxyethyl)aminouracil (II), 30.0 gm., 0.14 mole, was added, followed by 35.0 ml. of thionyl chloride. The reaction mixture was allowed to come to room temperature and was stirred at room temperature for 20 hours.

The reaction mixture was diluted with 1 liter of benzene and the resulting slurry was stirred for 1 hour. The solid was collected on a glass filter and washed well with benzene. It was dried at room temperature under vacuum; weight, 52.8 gm. The crude solid was recrystallized by dissolving it in about 400 ml. of boiling methanol containing 4 ml. of concentrated hydrochloric acid. The solution was dark and was treated with charcoal. To the hot solution was added 400 ml. of hot (70° C.) water and the flask was quickly cooled in ice. After the flask was chilled in ice for 4 hours, the crystals were collected and washed with cold water. The light-tan crystals were dried under vacuum at room temperature; weight, 22.85 gm. (65%), m.p. 203 to 206° C. (dec.) (block). This material was recrystallized by dissolving it in a slight excess of boiling acetone (about 1100 ml.), treating the solution with Magnesol and Darco-G-60, and filtering through a thin layer of Celite. The light, clear solution was concentrated at atmospheric pressure until crystallization began. The flask was cooled overnight at 0 to 4° C.; the crystals (needles) were collected on a filter and washed with a little acetone. They were dried under vacuum at room temperature; weight, 16.63 gm., m.p. 203 to 207° C. (dec.) (block). A second crop was obtained by concentrating the filtrate until crystallization began; weight, 3.48 gm., m.p. 203 to 207° C. (dec.) (block). The 2 crops weighed 20.11 gm. (47.2%). A third crop of somewhat less pure material was recovered by concentrating the mother liquor from the second crop. An analytical sample was prepared by recrystallizing a portion of the first crop from acetone; m.p. 198 to 204° C.

Analysis calculated for $C_8H_{11}Cl_2N_3O_2$: C, 38.11; H, 4.40; N, 16.67. Found: C, 38.54; H, 4.44; N, 16.83.

The ultraviolet spectrum in 0.01 N H_2SO_4 in 95 percent ethanol showed a maximum at 257 mμ–aM 5,675 and a broad flex at 300 mμ–aM 1,825.

The bishydroxyethylation of (I) as indicated in text-figure 1 proceeded smoothly with a yield of 67 percent, but the chlorination of (II) proved to be much more difficult. The usual conditions for reaction of thionyl chloride with bis(2-hydroxyethyl)amines—namely, with the reagent alone or in benzene under reflux—were completely unsuccessful.

After trying a variety of solvents, we found that the reaction proceeded smoothly in diethylene glycol dimethyl ether (Diglyme). In addition the temperature had to be kept below 40° C., or tarry materials, from which little product could be isolated, were formed.

The presence of a little water and alcohol in the solvent provided hydrochloric acid by reaction with the thionyl chloride. When (II) was subsequently added, its hydrochloride was formed, and the reaction proceeded more smoothly than when pure solvent and the free base were used. Under the conditions mentioned above, U-8344 (III) was obtained in 65 percent yield. Recrystallization of this material from acetone gave 57 percent yield of purified product, though this procedure did not appear to improve the physical constants significantly.

Toxicology and Pharmacology

The acute toxicity (LD50) was determined according to the method of Reed and Muench (*4*), with the use of young male rats of about 100 gm. average weight. The hepatotoxicity was indicated by the increase in sleeping time of mice receiving 100 mg. per kg. of hexobarbital, which is produced by a dose of the given drug based on 20 percent of the LD50 determined on the same strain and administered intraperitoneally.

The pharmacodynamic activities were evaluated in dogs weighing about 10 kg., which were anesthetized intravenously with pentothal and barbital. Autonomic activity was evaluated in terms of interaction of U-8344 with epinephrine, methacholine, histamine, atropine, tripelennamine, and tetraethylammonium standards. The dose of (III) was 1, 2, 4, and 8 mg. per kg., with the latter being the maximum static dose; the total dose was 15 mg. per kg.

The acute toxicity in young male rats (100–120 gm.) was found to be about 3.7 mg. per kg. intraperitoneally, 6.2 mg. per kg. subcutaneously, and 7.5 mg. per kg. orally. The drug was somewhat less toxic for older animals, but the oral toxicity remained about one half the intraperitoneal toxicity. Three daily doses of 0.5 mg. per kg. killed all rats of about 65 gm. average weight, while 1.0 mg. per kg. given on alternate days for 3 doses was tolerated by animals of the same weight range, indicating the importance of timing in achieving a proper dosage schedule and the cumulative effect of the drug. Rats tolerated 0.25 to 0.30 mg. per kg. daily for long periods of time, with only moderate toxic manifestations.

U-8344 (III) at one fifth the LD50 did not increase the sleeping time of mice under hexobarbital anesthesia, whereas chlorambucil, or *p*-bis(2-chloroethyl)aminophenylbutyric acid did increase it 100 percent. Fol-

lowing Plaa, Evans, and Hine (*5*), and Clarke, Reilly, and Stock (*6*), these data are taken to indicate that U-8344 has minimal hepatotoxicity.

No autonomic or cardiovascular effect was noted in the limited studies carried out on the pharmacodynamic activity of (III) in dogs.

Antitumor Activity

Weanling male rats of the Upjohn (S-D) strain were used in all experiments reported. They were kept under standard laboratory conditions in individual cages, and received a stock diet, prepared in our laboratory, until they weighed about 100 gm. At this time, Walker carcinosarcoma 256 was implanted into the right axillary region of each rat by a cell suspension of about 5 million cells in physiological saline.

Therapy with the drugs described was by intraperitoneal injection or by oral intubation, with a medium containing 9.5 percent ethyl alcohol in physiological saline. The stock solutions or suspensions were made up in 95 percent ethanol and diluted under aseptic conditions with 9 volumes of physiological saline just before the drugs were administered to the rats. Both U-8344 and chlorambucil have been found to be stable in ethanol for long periods, but unstable in aqueous solutions.

Therapy was begun either 24 hours after implantation of the tumor (indicated as T-1) or at a later date, as indicated in the tables. The time after tumor implantation is denoted by T-N, where N indicates the number of full days which have elapsed since the time of tumor implantation, *e.g.*, T-5 means the 5th daily anniversary of tumor implantation. Percent inhibition was calculated for each group on the basis of the average of 2 diameters of the tumors measured through shaved skin, and included those animals with no tumor. Regressions mentioned in the tables refer to complete disappearance of previously measurable tumors so that there was no palpable mass. In the instances where a sterile abscess or a small fibrotic mass persisted without showing growth and with no histologic evidence of tumor cells, the tumor was considered to have regressed.

The data shown in table 1 indicate that U-8344, when given intraperitoneally within 24 hours of implantation, completely inhibits the Walker tumor, and that the inhibition is effective by 3 doses of 1 mg., or 2 mg. per kg., or 2 doses of 4 mg. per kg., given on alternate days. The inhibition lasted for 10 days after dosing was stopped, at which time the experiment was concluded. There was no evidence of regrowth of tumors at the close of the experiment, though 1 small tumor nodule in each group receiving U-8344 remained for 7 to 10 days without change. Histologic examination of each of these revealed a sterile abscess surrounded by fibrotic mass.

The relationship of daily and intermittent dosing is shown in the data presented in table 2. Daily dosing for 17 and 14 days at 0.2 mg. per kg., beginning 2 days (T-2) or 5 days (T-5) after implantation, produces a small but definite inhibition, whereas dosing 3 times at 1 mg. per kg. on alternate days produces a high degree of inhibition and regression of the

TABLE 1.—Effect of intraperitoneal administration of U–8344 on growth of Walker carcinosarcoma 256 in male rats

Group	Survival	Weight gain in 15 days (*gm.*)	Tumor take	Tumor regression	Average tumor size*	Average tumor size†	Inhibition of tumor growth† (*percent*)
1. Tumor control	10/10	72	9/10	1/10	40 mm. (32–50)‡	32 mm.	—
2. U-8344, 1 mg./kg. on T-1, T-3, T-5	5/5	74	3/5	2/5	7	1	97
3. U-8344, 2 mg./kg. on T-1, T-3, T-5	5/5	68	4/5	3/5	6	1	97
4. U-8344, 4 mg./kg. on T-1, T-3	5/5	71	4/5	3/5	7	1	97

*Excluding regressions and "no takes."

†Including regressions, "no takes," and residual fibrotic nodules in groups 2, 3, and 4.

‡Range of size of tumors in control: Only one static tumor nodule remained in each treated group, each of which was found to be a fibrotic mass with a sterile abscess at center.

Note: 8 mg. per kg. of U-8344 on T-1 and T-3 caused death of all animals in groups 5 by T-6.

tumors, though appreciable toxicity was indicated in both cases by restricted weight gain. The total dose in groups 2 and 4, receiving the intermittent schedule, was 3 mg. per kg., while the dose in group 3 was 3.8 mg. and in group 5, 3.0 mg. per kg. Recovery from toxicity is better in the case of intermittent dosing, and the inhibition, which is long lasting, is in evidence 14 and 11 days after the last dose for groups 2 and 4, respectively. After this inhibition there was indication of regrowth of tumors. Other experiments have shown that many of the regrowths are still sensitive to the drug, and usually occur because of the formation of a fibrotic mass during regression, which, though small, often contains foci of tumor cells that have a poor blood supply.

Hematological studies indicated in table 3 were made on all animals in groups 2 and 4 of the experiment presented in table 2 on T-21. The averages given in table 3 indicate that these animals at this time showed a slight anemia, with those in group 4 being slightly more depressed than those in group 2. Although no normal animals were carried in this experiment, normal ranges of each blood value determined for animals of similar age and size carried in our laboratories are given for comparison. In other experiments in which U–8344 has been given for a longer period at 1 mg. per kg. intraperitoneally and in a larger total dose, marked leukopenia and anemia occurred during and immediately following the drug period. Therefore, it would appear that there was recovery in these groups from the probable initial hematopoietic depression.

The oral activity of the drug is shown by the data given in table 4. Here a comparison of the drug with chlorambucil, CB 1348, 4-{*p*-[bis(2-chloroethyl)amino]-phenyl}butyric acid, is included. The latter compound is one of the newer nitrogen mustard derivatives which has been found to have clinical value and which is highly active against the Walker tumor (*2*). In this experiment, U-8344 and chlorambucil were given to the rats after the tumors had been implanted 13 days, when all animals

TABLE 2.—Comparison of efficacy of U-8344 *versus* Walker carcinosarcoma 256 when given intraperitoneally, intermittently, and on a daily dosage schedule

Group*	Experimental day	Survival	Weight change during expt. period (*gm.*)	Number of measurable tumors	Average diameter of tumor (*mm.*)	Tumor inhibition (*percent*)
1. Control	20	10/10	157	10	50	—
	22	10/10	167	10	57	—
	24	5/5†	184†	5†	64†	—
2. U-8344	20	10/10	104	3	4	92
1 mg./kg. T-2	22	8/8‡	108	2	3	95
T-4	24	8/8‡	116	8	10	84
T-6						
total dose: 3 mg./kg.						
3. U-8344	20	9/10	84	9	28	44
0.2 mg./kg.	22	9/10	101	9	34	40
daily T-2 to T-19	24	9/10	108	9	40	30
total dose: 3.8 mg./kg.						
4. U-8344	20	10/10	99	3	5	90
1 mg./kg. T-5	22	8/10‡	108	3	3	95
T-7	24	8/10‡	119	7	8	88
T-9						
total dose: 3.0 mg./kg.						
5. U-8344	20	10/10	90	10	34	32
0.2 mg./kg.	22	10/10	109	10	39	32
daily T-5 to T-19	24	10/10	108	10	42	34
total dose: 3.0 mg./kg.						

*Ten animals per group initially.
†Five of 10 control animals used for further surgical experiments and hence eliminated from data.
‡Two animals in groups 2 and 4 were killed on T-21 for pathology. (*Cf.* table 3 for hematology.)

TABLE 3.—Hematology of rats of experiment shown in table 2 (data taken on T-21)

Group	Average hemoglobin (*gm.*)	Average hematocrit (*percent*)	Average BC	Differential count: Heterophils (*percent*)	Differential count: Lymphocytes (*percent*)
Group 2					
1 mg./kg. of U-8344 intraperitoneally T-2, T-4, T-6	8.9	36	9.5×10^3/cmm.	26	63
Group 4					
1 mg./kg. of U-8344 intraperitoneally T-5, T-7, T-9	7.5	36.0	7.7 "	35	56
Normal ranges*	12–15	40–45	5–10 "	10–40	60–90

*Values obtained from Dr. R. L. Johnston, Upjohn Biological Research Division, Pathology Department, and based on data compiled from other experiments involving a large series of normal controls.

had measurable tumors in the range of 5 to 35 mm. and the average diameter was 20 mm. This was the longest time we have been able to delay treatment and still find regression in a significant number of instances.

In group 2 the initial dose was 8 mg. per kg. of chlorambucil and the subsequent doses were 4 mg. per kg. each, with a total of 20 mg., while in group 3 the initial dose was 4 mg. per kg. and the subsequent 2 doses

TABLE 4.—Effect of oral administration of U-8344 on established Walker carcinosarcoma 256 (drugs dosed beginning T-13)

Group	Expt. day	Survival	Average weight* gain (*gm.*)	Average size of tumor (*mm.*)	Regression	Tumor inhibition (*percent*)
1. Tumor control†	13	9/9	—	19	—	—
	20	9/9	36	39	—	—
	31	9/9	92	59	—	—
	43	6/9	139	79	—	—
2. Chlorambucil	13	9/9	—	21	—	—
8 mg./kg.	20	8/9	20	26	—	33
T-13	31	8/9	77	10	3	83
4 mg./kg.	43	8/9	129	14	5	83
T-15, T-18, T-20	50	8/9	149	16	6	—
3. U-8344	13	—	—	22	—	—
4 mg./kg. T-13	20	8/9	6	17	3	56
2 mg./kg. T-18	31	8/9	63	0	8	100
T-22	43	8/9	120	1	7	99
	50	8/9	140	0	8	(100)
4. U-8344	13	—	—	21	—	—
1 mg./kg.	20	8/9	16	18	1	55
T-13, T-15, T-18	31	8/9	70	12	3	80
T-20, T-28	43	8/9	115	11	4	88
	50	8/9	131	12	6	—

*Weight gain calculated from T-13 when drug was started.

†Five of nine control animals dead on T-45; remaining animals moribund and killed for pathologic studies on T-45.

2 mg. per kg. each for a total dose of 8 mg. per kg. In group 4 the dose of U-8344 was 2 mg. per kg. given intermittently 5 times for a total dose of 10 mg. per kg. The dose schedules were chosen on the basis of previous experience both with regard to toxic manifestations reflected in weight loss or restriction of weight gain and antitumor activity. We have always preferred to evaluate drugs as to antitumor activity in growing rats when there is minimal weight loss directly after drug administration and good recovery of growth thereafter, with not more than about 10 percent mortality attributable to drug toxicity. For example, it was known that male rats tolerate orally 8 to 10 mg. per kg. of chlorambucil with a small weight loss and rapid recovery within several days, whereas 4 to 5 mg. per kg. causes no precipitous weight loss, but only a restriction of weight gain. Similarly, male rats can tolerate 3 to 4 mg. of U-8344 orally for 1 dose a week with good recovery of weight gain, whereas 2 mg. per kg. can be given several times a week with minimal or no weight loss and only a general restriction of weight gain.

This experiment was designed to investigate the oral activity against well-established tumors, since previous work had indicated the drug to be orally active against early tumors, and to compare the relationship of an initial high dose and subsequent lower doses of U-8344 with a schedule involving the lower dose each time. Chlorambucil was used as a standard for comparison and was administered as indicated under the most efficacious schedule of dosing which we had been able to devise.

The data indicate that U-8344 is as active as chlorambucil, which is one of the best drugs available against the Walker tumor, and that it is highly active when given orally against established tumors. It seems probable that the dosing schedule used in group 3 is preferable to that in group 4, since the regressions appeared earlier and were more extensive and lasting. Other data to be presented later indicate that the greatest efficacy with U-8344 is achieved when the drug is given orally at maximum tolerated doses every 4 to 7 days.

It is of interest to note that the regressions continued to occur after the drugs were stopped and that they held for long periods, up to 4 weeks in group 3, though there was regrowth in group 2. The animals receiving U-8344 showed no permanent damage during pathological examination at the termination of the experiment. Whereas only 1 animal died in each of the treated groups, more than 50 percent of the control animals were dead on the 45th day of the experiment, and all those remaining were moribund and had to be destroyed. The animals that died in the treated groups bore large, rapidly involuting tumors, and were lost early in the experiment. None of the control animals showed any sign of inhibition or regression.

DISCUSSION

The data presented here indicate that 5-bis(2-chloroethyl)aminouracil, U-8344, which is relatively easily prepared, is an active parenteral and oral antitumor agent, causing inhibition and regression of early and established Walker carcinosarcoma 256 in rats.

Other data to be presented later by us show that the compound is active in causing inhibition and regression of the Murphy-Sturm lymphosarcoma, Jensen sarcoma, and Guerin carcinoma in rats, as well as the Cloudman S91 melanoma and Adenocarcinoma 755 in mice (*3*). The wide spectrum of activity of the drug is further shown by the report of Evans and Mengel (*7*) of activity against Sarcoma 180 and Ehrlich's ascites carcinoma in mice and by the extensive studies of Lane and Kelly (*8*) in which the uracil mustard was shown to be highly active against 6 lymphoma and leukemias in mice and rats, and against mammary Carcinoma 241-6. The latter work showed that U-8344 was definitely superior to HN_2.

It is of importance to note that uracil mustard appears to be more efficacious when given intermittently at maximum tolerated doses than when given on a daily schedule at equal total doses. The fact that the drug has been shown to be active in causing regression of a wide spectrum of established transplantable tumors (*8*), coupled with the lack of pharmacodynamic side effects often associated with nitrogen mustard therapy, indicates the desirability of investigating the clinical usefulness of this drug.

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Biologic Studies on the Development of DON Resistance in a Mast-Cell Neoplasm of the Mouse [1]

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SUMMARY

The ascitic form of the mast-cell neoplasm P815 and the glutamine antagonist DON (6-diazo-5-oxo-L-norleucine), a growth inhibitor of P815, were used as a biologic system for studying the development of DON resistance. Titration studies were performed in which varying doses of ascitic tumor cells (10^2 to 10^7) were inoculated into untreated and DON-treated mice. Mice inoculated with 10^5 or more cells and treated with DON developed growing populations while under treatment, but consistently outlived the untreated control mice by more than 100 percent of the average survival time of controls. Some DON-treated mice inoculated with 10^2 or 10^3 cells did, however, survive indefinitely. A number of isolations of tumor cells were made from treated mice inoculated with the various cell doses. Characterizing these isolations for sensitivity or resistance revealed the following: Some cell populations were resistant and remained so; others, while showing resistance, lost this characteristic during serial transfer in untreated mice, and some were near-sensitive. Reconstruction studies were done by mixing various numbers of sensitive and known-resistant cells. Through this technique it was possible to detect, on the basis of survival time, as few as 10 to 20 resistant cells. From these experiments it was concluded: (1) Some cells sensitive to DON may be destroyed during DON treatment, but others persist as sensitive cells. (2) Resistance to DON may develop in these cells and hence during contact with DON. (3) Some resistant populations that are not stable are mixed populations (sensitive and resistant) in which sensitive cells apparently have a selective advantage over resistant cells during transfer through untreated mice.—J. Nat. Cancer Inst. 23: 163–181 1959.

MANY ANTIMETABOLITES have been shown to inhibit the growth of transplantable neoplasms in mice (*1*) but in most situations the grafted neoplasm, when established by an adequate number of cells, is not destroyed. Total destruction of grafts has been achieved (1) when a small number of cells are used to initiate the graft (*2, 3*), (2) when a particularly high pharmacologic level of the antagonist is reached (*4*), and (3) when possible immunologic differences exist between host and graft. Several biologic mechanisms offer some explanation why transplanted neoplasms survive and grow, with sustained treatment. The origin of antimetabolite-

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[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

resistant cells is one of these and is well documented (*1*). A second mechanism, not well defined at this time, is a temporary or physiologic adaptation of cells to the antagonist. Resistance achieved by this mechanism would not be expected to remain a stable characteristic in the absence of the inhibitor. Physiologic adaptation also might be attended by a burst of growth in the presence of the antagonist, once a pathway for relieving the block had been achieved. A third mechanism, persistence, may be postulated. Fundamental to this mechanism would be the innate potentiality for survival in the presence of the antagonist, since most of the therapeutic regimens of antimetabolites used in the various model experiments cannot be sustained because of host toxicity but are intermittent; persisting cells may find periods between treatments when limited growth is possible.

In regard to the emergence of resistant cells, there are two occasions in relation to treatment when the change from sensitivity to resistance may occur: (1) When a pre-established variation in the population exists—random events in the genetic apparatus (somatic mutation) in some members of a large population occur continuously in the absence of any exposure to the agent. [Law has provided experimental evidence on this point (*5*)]; and (2) when there are random nonspecific mutational events during exposure to the antagonist in viable inhibited cells.

Previously it was found that the DON-sensitive mast-cell neoplasm P815 (*2*, *6*) was a good model system for examining aspects of these problems. The important characteristics of the system were that resistance to DON [6-diazo-5-oxo-L-norleucine, an antagonist of glutamine which blocks an essential step in the pathway of purine ring synthesis *de novo* (*7*)] could be developed rapidly. The DON-R-II line of P815 was described as an example of the rapid emergence of a stable heritable resistant line (*4*). Experiences with P815 in earlier transfer generations were less favorable, because resistance developed only after several transfer generations in DON-treated mice. However, when it was found that resistance could be developed rapidly at the 44th-transfer generation, further studies were undertaken to examine some of the biologic characteristics of this event. This paper reports these studies.

MATERIALS AND METHODS

P815 arose in a 16-month-old male DBA/2 mouse painted with methylcholanthrene. The original neoplasm appeared as a large subcutaneous tumor which dissected into the sternum, with visceral dissemination to the spleen, liver, and lymph nodes. In a single transfer generation, at transfer 3, P815 was converted to a stable, nonbloody, ascitic neoplasm. P815 is routinely transferred at 10- to 14-day intervals by serial intraperitoneal passage of whole or diluted ascites and has been continuously

maintained and studied in (BALB/c × DBA/2)F_1 hybrid mice, 2 to 3 months of age.

All mice in these experiments were kept in plastic cages, 6 to 8 mice to a cage, and were fed Derwood pellets and tap water *ad libitum*. Both male and female mice were used, but no differences were noted in regard to the sexes. The F_1 hybrids were obtained from the Animal Production Unit, Laboratory Aids Division, National Institutes of Health.

DON was put in solution in an 0.2 M phosphate buffer at *p*H 6.0, and stored at −10° C. when not in use. Fresh stock solutions were prepared every 2 to 3 weeks. DON [4] was administered at the dose level of 0.125 mg. per kg. daily, 6 times a week, by inoculation into the subcutaneous tissues at the nape of the neck. This dosage schedule is well tolerated by the F_1 hybrids and treatment may be maintained continuously for a 2-month period if necessary. There is some toxicity accompanied by weight loss during prolonged treatment. Infrequently, when toxicity caused considerable weight loss, the treatment schedule was interrupted for 1- to 3-day periods, which permitted the animals to recover. There were a few early toxic deaths, but in general this was not an important consideration. Treatment was begun in most instances on the day after inoculation of ascites cells. In experiments with the effect of DON on graded cell inoculums, the treatment was begun at the time of tumor inoculation or the day after tumor inoculation. In a single experiment, treatment was begun on the 7th day.

The graded cell doses were made by a serial dilution technique from an original measured aliquot. Serial dilutions were made with chilled Locke's solution, and with separate serologic pipettes. All cells were inoculated within 30 minutes of harvesting. Total ascitic tumor-cell enumeration was performed by techniques previously described (*8*).

P815 has preserved most of the morphologic features described previously (*6*). There has been some loss of granules in the ascitic tumor cells, and some increase in the basophilia (Wright's stain) of the cytoplasm. Inoculation of ascitic tumor cells was followed by an outgrowth of cells on the peritoneum, with concomitant infiltration into the loose structures of the abdominal cavity. There was also visceral infiltration of the liver, spleen, and adrenals.

The experimental method to characterize the various lines of P815 developed in this study for DON sensitivity or resistance was the survival-time test. The average percent increase in survival time (APIST) was used as the index.

The APIST was calculated by subtracting the average survival time, in days, of the untreated group of mice from the average survival time, in days, of the DON-treated group, and dividing by the average number of days' survival of the untreated group. This value was multiplied by 100, thereby expressing it as a percentile.

The APIST value can be of use when survivors in treated groups are not encountered. The sensitive reaction is the one given by the stemline population. From transfer generations 44 to 62, with inoculums for

[4] Supplied by Parke, Davis & Company, Detroit, Mich.

10^5 stemline cells, the APIST for the stemline population ranged from 102 to 140, and the average was 127. For the three stable resistant lines (DON R-II, DON R-II continuous, and L2) the APIST ranged from 1 to 20.7, with an average of 5.9 for inoculums of 10^5 cells. An APIST value of 20 or below indicates a resistant state while the sensitive APIST value is considered to be 102 and above. A number of values fall between 20 and 102. Arbitrarily, values of 80 to 102 were considered near-sensitive, and 20 to 80 were considered intermediate. A fluctuation in the average survival time of untreated controls influenced this value, and it was necessary to have a number of determinations on a specific line to characterize more fully the response of that line.

RESULTS

The Response of Varying Cell Doses of P815 to DON

In a previous report the survival time of mice inoculated with cell doses of 10^5 to 10^6 of the mast-cell neoplasm P815 was reported (*2*). In addition, during the first 62 transfer generations, titration studies were performed, which are reported in table 1. Different inoculums from 10^7 to 10^2 cells were given in the individual experiments. In each instance there were 8 or more mice in a group. One group was treated with DON and a similar group was used as an untreated control. All recorded deaths followed tumor growth and infiltration. There were several 100-day survivors, in the treated groups, among the mice inoculated with 10^3 cells or less but not with higher inoculums. Untreated mice inoculated with 10^3 or 10^2 cells always developed growing P815 populations, and then died.

The Isolation of Cells for Studies of Resistance

All mice in which attempts were made to develop resistant lines are recorded in table 1. Each of the transfer lines is designated by a letter superscript above the recorded day of death for an individual mouse. In all, there were 15 isolations of populations taken from 13 mice. The isolations were usually selected from animals that had visible ascites. In experiments 1 and 2, only a single isolate was made. In the other two experiments, 3 and 4, there were 4 and 9 isolations, respectively.

Samples of ascites were removed from the living mouse by aspiration in a 1-ml. tuberculin syringe fitted with a 25-gauge needle. In some instances, cells could not be removed without previous injections of 0.5 to 1 ml. of sterile Locke's solution. The cells removed by aspiration were further diluted in test tubes.

Enumeration of tumor cells in the sample was then made in a hemocytometer, and the desired dose was given intraperitoneally to various groups of mice for a survival-time test. Three untreated mice, in a separate cage, were inoculated to establish a transplant line. The transplant line made from the isolate was independently maintained in

mice that did not receive DON. Serial transfer of the independent transfer line was made with an arbitrary dose of cells.

The results of these experiments are reported according to the experiment number in tables 2, 3, and 4.

Experiment 1.—The origin of the DON R-II line was described in a previous publication (*2*). Briefly, this line was isolated from a mouse on the 33d day after tumor inoculation (table 1). The mouse had been given 1,000 stemline cells. Daily treatments with DON began the day after tumor inoculation and continued for 6 days a week thereafter, for a total of 26 treatments. The mouse had obvious ascitic outgrowth on the 33d day and cells were easily withdrawn. The selection of this mouse may have been fortuitous because it was the first in the group to die. The next mouse died 7 days later, and 2 mice in the group survived over 100 days. Treated mice that survive 100 days with P815 have never subsequently developed tumor growth. The DON R-II line was used in the reconstruction studies reported later in the text.

For comparison, 2 sublines of DON R-II were studied. In the first, the cells were exposed to DON for only a single transfer generation, that is, during the 44th-transfer generation, and thereafter were transferred only in untreated mice.

A second line was initiated by continually passing DON R-II for 19 consecutive transfers through DON-treated mice (DON R-II continuous line).

The responses of both lines are given in table 2. It may be seen that with the single exposure line complete resistance was obtained during the course of the 33 days' residence in the DON-treated host. A titration study with different doses of cells was made in the 45th-transfer generation. It may be seen that, with the different cell doses, there was striking uniformity in the average time of death of the mice. One mouse inoculated with 100 cells and treated with DON failed to develop ascites and has survived indefinitely. It may be seen, also, that this line has been observed through 41 transfers after the single exposure, and resistance has persisted.

There was, however, some fluctuation in the APIST, which varied from 1 to 20.7. It may be noted, however, that these variations were not progressive and changes occurred from generation to generation. Most of the studies were done with inoculums of 10^3 cells. An acceleration in tumor growth rate reflected by a decrease in the average survival time of control mice was noted.

In the studies reported, the response of the continuous line of DON R-II has been more uniform. Here it was found that after 24 transfer generations through untreated mice, there was a decrease in the average survival time of controls. In 3 instances, the average survival time of DON-treated mice was less than that observed in controls.

Experiment 2.—Table 3 records the data on the A line, which was isolated from a mouse in experiment 2 (table 1). In this experiment, 10^5 stemline cells were inoculated into 3 groups of mice: (1) untreated con-

TABLE 1.—Effect of DON on the survival time of mice inoculated with stemline P815 cells

Transfer Cell			Control								DON treatment		DON treated*								Averages		APIST
			Day of death:								Day begun	Total daily	Day of death:										
Experiment	Generation	Dose	1	2	3	4	5	6	7	8	Last given	treatments	1	2	3	4	5	6	7	8	Control	DON	(*percent*)
1	44	10^5	17	17	17	18	18	18	18	19	1/36	29	32	33	33	33	39	39	39	39	17.7	35.8	102
		10^3	19	19	19	20	20	20	22	25	1/56	48	35†	42	42	43	46	49	S	S	20.5	42.8 + 2S	—
		10^2	20	22	22	22	22	25	25	27	1/56	48	43	43	46	S	S	S	S	S	23.1	44 + 5S	—
2	60	10^5	12	13	13	13	13	14	14	14	0/30	29	26	31	31	32	33	33	34	34	13.2	31.7 (d_1)	140
											7/25	17	23	23	23	24[A]	26	26	27	29	—	25.1 (d_7)	—
3	53	10^5	14	14	14	14	15	15	15	15	1/39	26	24	30	35	36[B]	37	37	40	42	14.5	35.1	142
		10^3	17	18	18	18	18	18	19	20	1/54	40	Tox	53[C]	54[D]	55[E]	63	73	73	S	18.2	61.8 + 1S	—
4	62	10^7‡	11	12	12	12	12	13	13	15	0/27	24	21	22	24[F]	26	27	28	28	29	12.5	25.8	106
													22	24	24	26	27	28	28	29			
		10^5	12	12	13	13	14	14	14	14	0/33	29	27	30	32[I]	32	32	34	34	35	13.2	31.7	131
													28	29	31	32	32	33	33	35			
		10^3	15	15	15	15	15	15	15	16	0/50	45	Tox	33	33	47	49	49[J]	57	60	15.1	45.5	201
													39[H]	41[K]	44	44	45[G]	45	47[L]	49			

*Letter following day of death indicates source animal for isolation of cells.
†Donor mouse for the DON R-II line.
‡1.18×10^5 cells inoculated.
See tables 2, 3, and 4 for details. Animals marked G, K were tapped on 2 occasions for cells.
"Tox" = premature death from toxic effect. "S" = survivor.

TABLE 2.—Effect of DON on the survival time of mice inoculated with the DON R-II line of P815

Transfer generation	Number of transfers: On DON	Number of transfers: Since last DON	Cell dose	Number of mice: Controls	Number of mice: DON	Survival time in days: Controls Average (*range*)	Survival time in days: DON treated Average (*range*)	APIST (*percent*)
Single exposure line*								
45	1	0	10^5		8/8	16.7(15–20)	17.1(17–18)	2.4
45	1	0	10^3		8/8	20.5(20–22)	21.5(20–23)	4.9
45	1	0	10^2		8/8	21.0(20–22)	22.8(18–27)†	8.5
46	1	1	1.15×10^3		8/8	19.8(19–23)†	23.2(20–25)	17.2
47	1	2	1.17×10^3		8/8	21.5(19–28)	21.8(19–23)	1.4
48	1	3	1.32×10^3		8/8	20.3(18–21)	24.2(21–26)	19.2
50	1	5	10^3		8/8	19.1(18–20)	23.0(21–35)	20.4
54	1	9	10^3		8/7	17.1(14–18)	19.8(18–22)	15.8
57	1	12	10^3		8/8	18.0(16–20)	18.2(16–20)	1.1
72	1	27	10^5		8/8	13.5(9–17)	16.3(16–18)	20.7
86	1	41	10^5		8/7	13.8(12–17)	15.4(15–16)	11.6
Continuous line‡								
62	19	0	10^5		6/6	17.6(14–23)	14.8(14–16)	<1:1
67	19	5	10^5		6/6	15.5(13–17)	13.6(13–15)	<1:1
69	19	7	10^5		6/6	13.3(12–15)	14.0(14)	5. 2
86	19	24	10^5		8/8	13.1(12–15)	12.5(12–14)	<1:1

*The single DON R-II donor mouse inoculated by 1,000 stemline cells (transfer-generation 44); cells isolated on 33d day post inoculation—26 daily treatments of DON had been given.
†One mouse did not develop growth and survived 100 days observation.
‡The continuous line was carried through DON-treated mice for 19 transfer generations.

TABLE 3.—The effect of DON on the survival time of mice inoculated with various isolates from experiments 2 and 3

Line	Number of transfers: Day isolated / Died	Number of transfers: Number daily DON treated	On DON	Since last DON	Cell dose inoculation	Number mice in group	Control: Average (*range*)	DON treated: Average (*range*)	APIST (*percent*)
Experiment 2									
A	23/24	14	1	0	9.6×10^5	6/6	13.1(12–14)	14.5(12–16)	10. 7
	—	—	1	4	10^5	6/6	12 (12)	17.8(16–19)	48. 3
	—	—	1	7	10^5	6/6	13 (13)	19.5(19–21)	50. 0
	—	—	1	9	10^5	6/6	13.3(12–14)	19.3(18–21)	45. 1
	—	—	1	18	10^5	6/6	10.5(10–11)	24.0(22–26)	128. 0
Experiment 3									
B	32/36	24	1	0	3.35×10^5	6/6	17.5(16–23)	18.5(18–19)	5. 7
	—	—	1	4	10^5	6/6	13.5(13–14)	21.3(20–23)	57. 7
C	45/53	30	1	0	4.4×10^5	8/8	16.1(15–19)	25.2(21–28)	56
D	54/55	40	1	0	10^5	6/6	15.6(15–17)	20.8(19–23)	33. 3
E	54/55	40	1	0	10^5	6/6	15.1(14–20)	18.5(17–20)	22. 5
	—	—	1	5	10^5	6/6	14.1(14–15)	18.8(17–20)	33. 3
	—	—	1	7	10^5	6/6	14.1(13–15)	19.5(19–21)	38. 3
	—	—	1	8	10^5	6/6	13.6(13–15)	20.5(20–21)	50. 6
	—	—	1	16	10^5	6/6	13.8(13–15)	26.1(22–28)	92

TABLE 4.—The effect of DON on the survival time of mice inoculated with various isolates from experiment 4

Line	Day isolated / Died	Number daily DON treatments	Transfer generation: On DON	Transfer generation: Since last DON	Cell dose inoculated	Number of mice: Controls / DON	Survival time in days: Control Average (*range*)	Survival time in days: DON treated Average (*range*)	APIST (*percent*)
F (10^7)	23/24	20	1	0	10^5	6/6	17.1 (15–20)	15.5 (14–17)	<1
(10^7)	—	—	1	4	10^5	6/6	14.8 (14–16)	16.1 (15–17)	8.8
	—	—	1	7	10^5	6/6	13.6 (12–17)	17.3 (17–18)	19.8
G (10^3)	25/45	20	1	0	10^3 *	6/6	15 (15)	27.8 (25–31)	85.5
	44/45	37	1	0	10^5	6/6	15.3 (14–16)	24.1 (23–25)	57.5
H (10^3)	25/39	22	1	0	10^5	6/6	14.6 (14–15)	22.3 (19-24)	52.6
I (10^5)	25/32	22	1	0	10^5	6/6	15.5 (14–20)	18.6 (17–21)	20.0
J (10^3)	30/49	25	1	0	10^5	6/6	16.5 (16–17)	32.0 (30–34)	94
K (10^3)	30/41	25	1	0	10^5	6/6	15.1 (15–16)	23.3 (21–26)	53.6
(10^3)	41/41	35	1	0	10^5	6/6	14.5 (14–15)	21.5 (18–23)	48.1
L (10^3)	44/47	37	1	0	10^5	6/6	15.0 (14–16)	21.6 (20–23)	44.0
(10^3)	44/47	37	1	0	10^5	6/6	14.1 (13–15)	21.5 (21–23)	52.5
(10^3)	44/47	37	1	5	10^5	8/8	13.1 (12–14)	20.7 (20–27)	58
(10^3)	44/47	37	1	15	10^5	8/8	14.5 (13–15)	26.8 (23–31)	84.8
L2 (10^3, 10^5)	19/21	15	2	0	10^5	6/6	15.3 (15–16)	15.5 (14–18)	1.3
(10^3, 10^5)	—	—	2	3	10^5	8/8	14.6 (14–16)	15 (14–16)	2.7
(10^3, 10^5)	—	—	2	7	10^5	6/6	13.5 (11–15)	14.3 (13–15)	5.9
(10^3, 10^5)	—	—	2	14	10^5	8/8	15.6 (15–18)	17.5 (17–18)	12.1

* = 9×10^3.

trols, (2) mice treated with DON beginning when tumor cells were inoculated, and (3) mice treated with DON on the 7th day after tumor inoculation. Cells were removed from 1 mouse in this 3d group on the 23d day, and the A line was initiated. At this time the DON-treated mice in this group had ascites. On direct isolation the A line was resistant (table 3). After 18 transfers through untreated mice, however, the A line became DON-sensitive.

Experiment 3.—In table 3 are also included the studies on 4 isolations from experiment 3 (table 1). In this study, the 2 cell doses were 10^5 and 10^2. Line B was isolated at the 32d day from a DON-treated mouse originally inoculated with 10^5 cells. The APIST in the first-transfer generation was 5.7 but, after 4 transfer generations through untreated mice, changed to 57.7.

Three other lines isolated in this experiment from DON-treated mice, which were inoculated originally with 10^2 cells, were isolated at days 45 (line C), 54 (line D), and 54 (line E). Line E, which was resistant on initial isolation (APIST 22.5), gradually became near-sensitive after 16 serial transfer generations through untreated mice. Line C and D showed an intermediate resistance on initial isolation and were not carried further.

Experiment 4.—In this experiment there was a total of 9 isolates from 7 donor mice. One isolate originated from a DON-treated mouse originally inoculated with 10^7 cells (the F line). One isolate originated from a mouse originally inoculated with 10^5 cells (the I line). All others were from mice inoculated with 10^3 cells, and in 2 of these mice, 2 isolates each were taken from the same mouse, but at different days. The G line was isolated on the 25th and 44th days; the K line on the 30th and 41st days. There was, respectively, an 11- (line K) and 19- (line G) day difference between the time of isolation for the 2 pairs. Line G on direct isolation on the 25th day gave a near-sensitive APIST value (85.5), and later, on the 44th day, gave an intermediate value (57.5). Line K showed an intermediate resistance on both occasions.

In these 9 isolates, the lines showing resistance were derived from mice inoculated with the highest doses of cells (lines F and I). None of the isolates from the mice inoculated with 10^3 cells were resistant. Line J, which was isolated at the 30th day from a DON-treated mouse inoculated with 10^3 cells, was near-sensitive—the APIST value was 94. A similar response was seen on the 25th day for the isolate of line G.

The L line isolated from a mouse originally inoculated with 10^3 cells was initially isolated on the 49th day and gave an APIST value of 44 (intermediate resistance); after 15 transfers through untreated mice, the APIST value became near-sensitive (84.8). However, when the direct isolate (obtained on the 44th day) was transferred to DON-treated mice for 1 additional transfer generation (a period of 19 days), a stable resistant line was obtained—the L2 line. This line remained stable, for resistance, for 14 transfers through untreated mice.

The reversion to sensitivity of some of these lines (A, E, and L) could be the result of a change in the character of resistant cells. Striking

stability of some resistant lines (DON R-II and L2) suggests, however, that the cause of the reversion in lines A, E, and L might be explained in another way, essentially, by a change in the population. A premise here is that sensitive cells possess a growth advantage over resistant cells in untreated mice.

Reconstruction Experiments

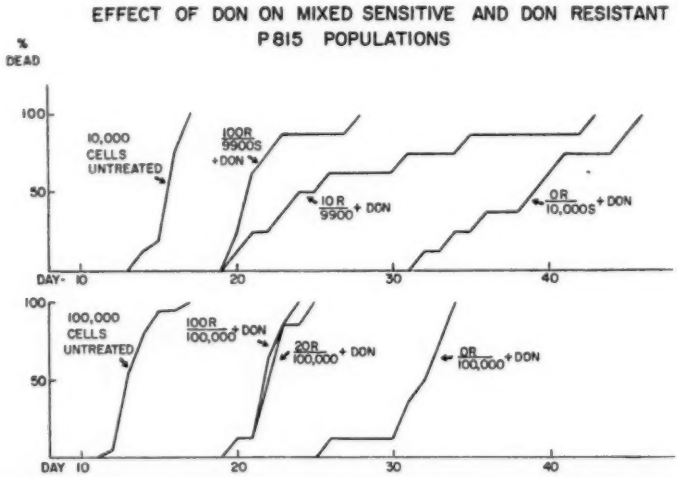
To determine the effect of DON on mixed populations of cells, it was necessary to have a resistant cell type which possessed the same growth rate as the stemline population. The DON R-II line was used for this purpose. In these studies, stemline cells of P815 were mixed with varying proportions of cells from the DON R-II line. (DON R-II single exposure: 9th- through the 12th-transfer generations.) This line was carried in mice that were not treated with DON, and thus these cells had resided for only 1 transfer generation in DON-treated hosts (table 1). The results of the experiments are presented in table 5 and text-figures 1 and 2.

TABLE 5.—The effect of DON on the survival time of mice inoculated with mixed populations of P815 cells

Experiment	Number of cells inoculated		Transfer generation		Survival time in days		
		Resistant			Control	DON	
	Total	Stemline	S	DON R-II	Average (*range*)	Average (*range*)	APIST (*percent*)
5	1,000	10 / 990	53	54	18.5 (17–20)	26.3 (22–35)	42.2
6	10,000	0 / 10,000	55	—	15.4 (14–17)	39.1 (32–46)	153
	10,000	100 / 9900	55	56	16.5 (16–17)	22 (20–28)	33.3
	9,910	10 / 9900	55	56	15.8 (15–16)	27.8 (20–43)	76
7	100,000	0 / 100,000	60	—	13.2 (12–14)	31.7 (26–34)	140
	101,000	1,000 / 100,000	60	56	14.7 (13–17)	20.1 (20–21)	36.7
	100,000	100 / 100,000	60	56	13.7 (13–15)	22.2 (20–24)	62
	100,020	20 / 100,000	60	56	13.5 (13–15)	22.2 (20–25)	64.5
8	100,000	0 / 100,000	61	—	13.7 (13–15)	31.5 (22–35)	130
	101,000	1,000 / 100,000	61	57	14.7 (13–16)	18.7 (18–20)	27.2
	1,000	1,000 / 0	61	57	18.0 (16–20)	18.2 (16–20)	1.1

In experiment 6, three different groups were observed: (1) 10^4 stemline cells; (2) 100 DON R–II cells plus 9,900 stemline cells; and (3) 9,900 stemline cells plus 10 DON R-II cells. The presence of the resistant cells was readily demonstrated by the survival time of the DON-treated

mice. In each group the survival time was less than that observed for pure stemline cell transfers. The second group, with 100 resistant cells added, reduced the survival time of the DON-treated group from the expected average number of days (39.1) seen with the stem line to 22 days. In the third group in which only 10 cells were inoculated, the survival time was reduced to the average of 27.8 days. It may be seen from text-figure 1, where the time of death of individual mice is recorded on a graph, that the curve for the DON-treated group of mice inoculated with 9,900 stemline cells and 10 resistant cells appears to be bimodal; that is, 5 of the 8 mice in this group died between the 20th and 26th days, and the last 3 died between the 31st and 43d days. This result suggests that the 10 cell inoculums probably did not successfully survive in the last 3 mice. In experiment 7, also recorded in text-figure 1, in which 20 resistant cells were inoculated with 100,000 stemline cells, a bimodal curve was not obtained. The inoculation of 20 resistant cells was effective in all mice.

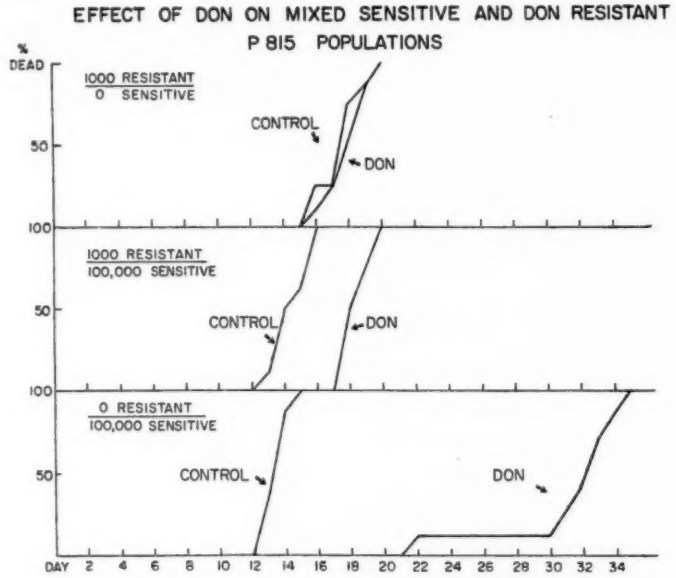


TEXT-FIGURE 1.—Reconstruction experiments. Stemline cells (S) were mixed with resistant (DON R–II) cells (R) in varying proportion. The figure represents a survival-time curve.

It must be remembered that these small inoculums are actually calculations from serologic dilutions and are subject to error. To perform such experiments accurately, microscopic identification of the cell number in a given sample would be required.

The results of experiment 8 are reported in text-figure 2 in which an attempt was made to show that the survival time of a mixed population inoculated into DON-treated hosts is a function of the number of resistant cells initially introduced. It may be seen that 10^5 stemline cells inoculated into untreated mice killed their hosts on an average of 13.7 days, whereas, 10^3 DON R-II cells killed their hosts on an average of 18.7 days.

In the mixed population, when DON was given and presumably inhibited the stemline population, the average time of death of the group was 18.2 days, which is similar to that observed with 10^3 DON R-II cells, in both treated and untreated hosts. In every instance, it was possible to detect the presence of resistant forms in the various combinations on the basis of survival time.



TEXT-FIGURE 2.—Reconstruction experiment.

Response of Isolates Obtained From Mice With 7- to 8-Day-Old Ascitic Tumor-Cell Populations, Treated With Short-Term Courses of DON

Isolates were obtained from large populations of stemline cells after short-term treatment schedules with DON (table 6). Stemline cells, 10^5 to 10^6, were inoculated into groups of mice and allowed to grow for 7 to 8 days, when one group was killed and total ascitic tumor-cell counts were made. After the initial control study, the remaining mice were put into control and treated groups, and ascitic tumor-cell counts were made from 2 to 6 days after the initiation of DON treatment. Enumeration of cells was made by lavaging the opened peritoneum and its contents in 3 separate beakers containing Locke's solution. The washings were then combined and the counts made. When a given mouse was selected for isolation, an aliquot of cells was preliminarily withdrawn with a sterile needle and syringe to prevent contamination of the sample.

TABLE 6.—The effect of DON on total ascitic tumor-cell numbers after DON treatment

Experiment	Cell dose / Stemline / DON R-II	Day DON began—No. of daily treatments	Day of harvest	Total ascitic tumor cells in millions†										Average crude mesenteric weight (*mg.*)	
				Control					DON-treated					Control	DON
9	8×10^5	7/0	7	388	314	304	266	189	—	—	—	—	—	588	—
	0	7/3	10	124	106[a]	97	74	64	166	63[b]	58	40	0	1520	473
		7/6	13	110	110	101	88	64	44	15[c]	8	7	3	2730	359
10	1.02×10^6	8/0	8	432	399	348	332	276	—	—	—	—	—	791	—
	0	8/2	10	538	294	291	153	73[d]	284	272	242	59[e]	36	1046	664
		8/2*	10	—	—	—	—	—	344[f]	251	229	214	151	—	683
		8/5*	13	—	—	—	—	—	257	170	170	168[g]	120	—	581
		8/5*	13	—	—	—	—	—	87[h]	77	73	59	48	—	376
		8/8	16	—	—	—	—	—	—[i]	—	—	—	—	—	—
11	10^5	7/0	7	224	196	192	113	—	—	—	—	—	—	547	—
	0	7/3	10	488	460	352	276	—	21	8	6[j]	2	—	808	366
12	9.1×10^4	7/0	7	208	142	116	103	—	—	—	—	—	—	517	—
	10^3	7/3	10	423	400	252	240	—	23	6[k]	3	1	—	844	434
	9×10^4	7/0	7	160	151	126	69	—	—	—	—	—	—	715	—
	10^4	7/3	10	473	367	331	251	—	71	49[l]	5	4	—	762	416

*The dosage of DON was 0.375 mg. per kg. for these mice.
†Small letters indicate donor mice for isolates.

The total ascitic tumor-cell counts obtained from control mice showed some variation from experiment to experiment. For example, in experiment 9, the control group of mice killed on day 7 had the greatest ascitic tumor-cell counts; thereafter there was a decline. This same general trend was seen between the 8th and 10th days in experiment 10. In experiments 11 and 12, there was an increase in the total number of tumor cells between the 7th and 10th days.

It may be seen also from table 6, in experiments 9 and 11 with DON-treated groups, that there was a definite decrease in the total tumor-cell counts after DON treatment. This degree of reduction was not observed in experiment 10, in which DON treatment was initiated on the 8th day. In experiment 10, however, some decrease in tumor-cell numbers was observed in mice treated with 0.375 mg. per kg. in daily doses at the 5th day.

The crude mesenteric weight was obtained by stripping the gut from the sigmoid region to the pylorus and removing the mesentery, which included the mesenteric lymph node. This index, an indication of the infiltrative growth capacity of neoplasm P815, progressively increased in controls in all experiments and consistently decreased in treated mice.

In experiment 12 (table 6) artificially mixed populations were used. DON treatment which began on the 7th day caused a reduction in the total tumor-cell number and in the crude mesenteric weight.

The isolates obtained from the mice indicated by a smaller letter in table 6 were transplanted to new mice. Survival-time studies on these isolates were made and recorded in table 7. It was found, on the basis of the APIST, that isolates made from stemline populations 2 to 3 days after the initiation of DON (isolates b, e, f, and j) were sensitive (j) or

TABLE 7.—Survival-time studies of mice inoculated with isolates from established populations treated with short courses of DON*

Experiment	Isolate	Number of daily DON treatments: Day isolated	Transfer generation	Cell dose $\times 10^6$	Survival time in days: Control Average (*range*)	Survival time in days: DON-treated Average (*range*)	APIST (*percent*)
9	a	0/10	54	0.87	16.7 (16–19)	38.1 (31–44)	128
	b	3/10	54	0.72	18.7 (16–26)	35.1 (33–42)	87
	c	6/13	54	0.25	19.5 (16–23)	28.1 (27–32)	44
10	d	0/10	59	0.1	12.5 (12–14)	27.5 (22–31)	120
	e	2/10	59	0.1	14.3 (14–15)	25.7 (22–29)	79.6
	f†	2/10	59	0.1	13.8 (13–15)	27 (25–29)	95.0
	g	5/13	59	0.1	14.1 (14–15)	26.2 (24–28)	85.6
	h†	5/13	59	0.1	13.8 (13–14)	25.7 (22–30)	86.1
	i	8/16	59	0.1	15.3 (14–19)	21.6 (18–25)	41.2
11	j	3/10	55	0.1	19.1 (17–20)	49.1 (43–55)	157
12	k‡	3/10	—	0.1	16 (15–17)	17.1 (16–19)	6.9
	l‡	3/10	—	0.1	16.7 (15–21)	17.3 (12–27)	3.6

*Each group of controls or treated mice contained 8 mice.
†DON dosage 0.375 mg. per kg.
‡Obtained from mixed populations.

near-sensitive (b, e, and f). Isolates b, e, and f differed from control isolates (a and d), however, indicating some resistant cells were present. It might be expected that DON should eliminate some sensitive cells and concentrate resistant cells. The individual donor mice for isolates b, e, and j showed that reduction in the total population had occurred. This was not true with isolate f.

Isolates from mice obtained at days 5 to 8, isolates c, g, h, and i, were not consistent. The 5th day isolates (g and h) from experiment 10 resembled the 2- and 3-day isolates by remaining near-sensitive in response to DON. The other 2 (c and i) revealed an intermediate resistance.

Particularly striking, however, were the results obtained in experiment 12, in which a mixture of stemline and resistant cells was used, since here it was seen that purely resistant isolates (k and l) were obtained.

DISCUSSION

Two general observations were made from experiments in which varying doses of stemline cells were inoculated into mice. Indefinite survival of DON-treated mice occurred only in a few inoculated with 10^2 or 10^3 cells (table 1). Populations on initial isolation that showed resistance were isolated from some DON-treated mice in all cell dosage groups. Subsequent study of certain isolates, after serial transfer through untreated mice, revealed changes in character. Lines A and E, though initially resistant after 14 transfer generations in untreated mice, reverted to sensitivity. Line L, initially intermediate, also became sensitive. A stable intermediate line was not found.

Two postulates may be advanced to explain why DON R-II and L2 did not subsequently revert to sensitivity, while lines A, E, and L did: (1) Cells from lines A, and E, while purely resistant, underwent an independent change to sensitivity. (2) Lines A, E, and L were mixed populations containing both sensitive and resistant cells; the sensitive cells outgrew the resistant during transfer in untreated mice. If the first postulate is accepted, the second becomes a necessary adjunct to it.

The second postulate is favored for several reasons: (*a*) It is quite apparent that DON does not eliminate all sensitive cells. Examples are to be found in lines G and J isolated from DON-treated mice on the 25th and 30th days, which gave near-sensitive APIST values (85.5 and 94). Further, DON failed consistently to eliminate all cells from the peritoneal cavity of mice with established populations, and sensitive isolates were recovered from some of these mice (tables 6 and 7). (*b*) The populations that reverted were mixed from the beginning as observed in line L2, a line afforded an additional 19-day period of selection in a DON-treated mouse, which did not revert, while the L line did.

It may be argued that the acquisition of resistance, as expressed by the behavior of the populations A and E, was not the result of a "genetic" change but due to temporary physiologic resistance (adaptive). While this cannot be disproved, the slowness of the reversion, which requires many transfer generations for full development, is not characteristic of this type of adaptive change. Then too, one might expect physiologic adaptation to be a general characteristic of all cells. This was not found to be true for lines G and J, which were sensitive on direct isolation.

The existence of genetically different cells that are DON-sensitive but capable of physiologic adaptation, as opposed to sensitive cells incapable of adapting, cannot be ruled out.

The evidence obtained from survival-time data, as well as that obtained from treating established stemline populations (7- to 8-day-old) with DON, does not indicate the rapid development of physiologic adaptation.

Some sort of adaptation must be considered, however, because some cells are destroyed by DON as evidenced from the total ascitic tumor-cell counts after DON treatment. Much work will be required to establish the nature of this type of adaptation.

Another question involved in the interpretation of the experiments is whether the stemline population between the 44th- and 62d-transfer generations contained resistant cells. To explore this point a survival-time study of intentionally mixed populations was used (table 5 and text-figs. 1 and 2). Ten to 20 resistant cells added to 10^4 to 10^5 stemline cells were readily discerned in survival-time curves—the survival time of mice was greatly reduced. It is not known how less than 10 resistant cells in a population of 10^5 stemline cells might shape the survival-time curve or whether the survival-time curves of DON-treated mice inoculated with 10^5 stemline cells are actually a function of both resistant and sensitive cells.

Some simple calculations, however, suggest a resolution to the question. If resistant cells were present in a stemline population, their distribution should be proportional to the total number of cells inoculated; for example, experiment 1 (table 1), in which the DON R-II line was isolated from a mouse inoculated with 10^3 cells. If 10^3 cells inoculated into this mouse contained a single resistant cell and, likewise, a resistant cell was found in each of the 6 mice that developed ascites and died while under DON-treatment, there would be a minimum of 6 resistant cells for 8,000 cells or 600 for 800,000. Each mouse inoculated with 10^5 cells would have received 75 resistant cells. The actual survival-time curve does not resemble that observed with the reconstruction experiments. This same argument may be repeated for other experiments, with similar conclusions, namely, that the resistant cells appear to arise after contact with DON.

The mice (table 1) that developed populations of P815 cells did not do so necessarily, if at all, because the stemline population contained resistant cells; nor was there a complete elimination of sensitive cells. Resistance appears to develop, however, more readily in mice inoculated with larger populations. Of the 15 isolates made (table 1), 44 derived from mice

inoculated with 10^5 or more cells on initial isolation were resistant (A, B, F, and I); whereas, only 2 of 11 of the others (mice inoculated with 10^2 or 10^3 cells) were initially resistant. Lines G and K are represented by 2 isolations each: line G was isolated from the same mouse at days 24 and 44 and line K at days 30 and 41. Dramatic changes in character were not seen, which indicated the resistant cells were emerging slowly and not overrunning the population in these cases.

In general the findings in these experiments may be summarized as follows: DON, a growth inhibitor of P815, may cause the death of some but not all cells of the stemline population. In fact, some sensitive cells survive and probably grow very slowly in the presence of DON. These cells provide a population in which a change to resistance may occur in one of the members. Resistant cells, when selected in DON-treated mice for sufficient periods (sometimes requiring additional transfers through DON-treated mice), remain resistant as do their progeny. If, however, the selection is incomplete, and both sensitive and resistant cells are obtained in a population, sensitive cells possess a growth advantage over resistant ones in untreated mice, and the populations revert to the sensitive state.

It cannot be concluded that DON specifically directs a change from sensitivity to resistance. The data do emphasize the importance of survival, and retention of the sensitive characteristics, with this inhibitor. This phenomenon has been called persistence. Persistence in bacteria has been described by Bigger (*9*). Goldin *et al.* described a line of L1210 that retained sensitivity to amethopterin after an extensive course of amethopterin treatment (*4*).

Potter and Law described the development of delayed recurrences in the plasma-cell neoplasm 70429 after extensive azaserine treatment. Some of these delayed recurrences were azaserine-sensitive (*8*).

Klein and Klein have used reconstruction experiments in some of their work dealing with amethopterin resistance (*10*). The relationship of the present findings to the problem of amethopterin resistance, which develops after several transfers through amethopterin-treated mice, will be the subject of a later paper.

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Virus of Avian Myeloblastosis. XII. Chemical Constitution [1, 2, 3]

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SUMMARY

Analyses were made of the chemical constitution of the virus of avian myeloblastosis. Studies made by microchemical techniques on samples of purified viruses from individual chickens yielded the values of nitrogen, 9.9; phosphorus, 1.27; pentose nucleic acid, 2.17; and lipide, 35 percent, respectively. No evidence was seen of deoxypentose nucleic acid. The content of phosphorus in excess of that bound in the nucleic acid suggests that much of the lipide may be in the form of phospholipide. Within the limits of the methods employed, the results indicated high uniformity in constitution of specimens from different birds, which corroborates evidence of the homogeneity of purified virus preparations previously obtained by analytical ultracentrifugation and electrophoresis. The findings show that in chemical constitution the virus of avian myeloblastosis does not differ in principle from that of filterable agents causing other diseases in man and animals.—J. Nat. Cancer Inst. 23: 183–197, 1959.

UNTIL RECENTLY, only one virus associated with the occurrence of malignancy had been obtained in a state of homogeneity suitable for a meaningful analysis. That agent, the virus responsible for the papillomatosis of rabbits (*1*) which progresses to epidermal carcinomatosis (*2*), can be isolated in preparations of high purity from the warts of cottontail rabbits by simple centrifugal procedures (*3*). The papilloma virus had a well-established experimental basis, with respect to the manner of occurrence and from the results of sedimentation and electrophoretic and electron micrographic studies, for justifying the judgment that the preparations obtained from pools of warts from various cottontail rabbits and subjected to analysis were homogeneous and highly uniform from one specimen to another.

The virus of avian myeloblastosis (*3*, *4*), an agent directly responsible for neoplastic disease, has become available for analogous studies. The virus occurs in very high concentrations (*5–7*) in the plasma of some

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[3] The results of the work described here were reported briefly at the Fourth International Congress for Biochemistry at Vienna, September 1–6, 1958.

chickens with myeloblastosis from which it can be recovered by repeated centrifugal fractionation. Although plasma is not a complex medium with respect to tissue elements or debris, as extracts of fleshy tumors are, it does contain a population of nonviral amorphous particles about the size of the virus, which have not been separated from the agent by alternate high- and low-speed centrifugation. Because of the physical characteristics of the virus, principally variation in size of the virus particles, and the high degree of light scattering and other properties of the more concentrated preparations, the usual criteria of analytical sedimentation (*8*) and electrophoresis (*7*) are not as fully applicable to the knowledge of the state of homogeneity as in the case of the papilloma virus. Nevertheless, information gained by electron micrography, together with other data, has indicated that preparations containing only traces of extraneous particles can be obtained by use of plasmas of the highest virus content (*9*).

It has been the usual procedure, as a matter of necessity, to analyze other viruses obtained from pools of material from several hosts. With the agent of myeloblastosis, however, because of access to newer ultramicrochemical techniques and to the richness of the virus in the plasma of diseased birds, the present investigations have been made in most instances with the product obtained from a single host. Thus, many of the data are significant not only for the understanding of the constitution of the virus but also for use as another criterion of uniformity of the material recovered from the plasmas of different birds. The procedure and the results of the analyses on such specimens of myeloblastosis virus are described in the present paper.

MATERIALS AND METHODS

The virus under investigation was the BAI strain A, which was derived initially by Hall, Bean, and Pollard (*10*) from chickens with neurolymphomatosis. These authors studied various manifestations of the disease in chickens and in chick embryos. Subsequently, Dr. E. P. Johnson studied the disease in chickens (*11*) and Dr. Johnson furnished this laboratory with a single chicken that had been inoculated with virus material of this strain which he had obtained directly from Hall, Bean, and Pollard. From this bird, an agent (*4*) was derived which has induced a highly malignant state of myeloblastosis without evidence of the occurrence of the related leukemia, erythroblastosis. There is much reason to assume from the results of long study, therefore, that the myeloblastosis virus analyzed in the present work represents an essentially pure strain of virus (*4*) which is different from the agent of erythroblastosis.

Virus for analysis was obtained from White Leghorn chickens of line 15, which was established (*12*) at the Regional Poultry Research Laboratory, East Lansing, Michigan. Birds were inoculated intravenously at 3 or 10 days of age with 0.1-ml. volumes of filtered plasma or suitable dilutions of such plasma from previously diseased chickens. Blood was drawn by

heart puncture into a solution of heparin (0.5 to 1.0 mg. crystalline heparin per ml. of blood) in tubes immersed in an ice bath. Cells were removed by centrifugation for 20 minutes at 1,000 × g, and the plasma was pipetted off, frozen, and stored at −78° C. until used. Ordinarily, about 3 to 10 ml. of plasma were obtained from the small chicks used. The birds were in a fasted state, since they ate little in the later stages of the disease. Plasma for an experiment was thawed under tap water (20° C.), rechilled in an ice bath, and centrifuged for 15 minutes at 2,500 × g. The virus preparations were kept at 0° to 7° C. during application of the purification procedures. In some cases, the plasmas were passed through Selas 015 filters (*13*). The virus particles were sedimented from plasma in an angle rotor spun in a vacuum chamber, usually at 15,000 × g for 30 to 45 minutes. Centrifugation for 30 minutes in the same gravitational field was used to recover the virus from suspensions in water or saline solution. Small variations from these conditions had no noticeable effect. The first pellet was dispersed in calcium-free Ringer's solution and centrifuged for 10 minutes at 2,500 × g. After a second high-speed centrifugation, the pellet was resuspended in water or Ca-free Ringer's solution, and the cycle of low- and high-speed spinning was repeated for a total of 4 or 5 cycles. The last high-speed pellet was taken up in a small volume and, after centrifugation at low speed, the suspension was parceled in the desired volumes and used immediately or frozen and stored at −78° C. When virus from several plasmas was pooled, the mixing was effected *after* the first cycle of centrifugation. The particles were readily redispersible in distilled water but less so in Ca-free Ringer's solution.

All reagents were of commercial quality except as noted. The chloroform and methanol used for lipide extraction were spectrophotometric grade, and the ethanol was treated with lead oxide and redistilled.

Total nitrogen was determined by the method of Grunbaum, Schaffer, and Kirk (*14*, *15*) and *total phosphorus* by the techniques of Schaffer, Fong, and Kirk (*16*).

Lipide and nucleic acid were measured by modification and combination of Sperry's (*17*) adaptation of the lipide method of Folch *et al.* (*18*), and the nucleic acid procedure of Scott, Fraccastoro, and Taft (*19*). An aliquot of virus suspension, usually 50 μl., was dried in a 6 × 50-mm. culture tube over silica gel in a vacuum at 2° C. It was extracted twice with 50-μl. portions of cold 0.3 M perchloric acid. These extracts were combined as the *acid-soluble fraction* and stored at −22° C. The residue was then extracted once with 50 μl. of 80 percent methanol, twice with 50 μl. of a mixture of 2 parts chloroform and 1 part methanol, and once with 50 μl. ethanol. These extracts were combined as the *lipide fraction* and stored at −22° C. All extractions to this point were done at 0° to 4° C. Fifty μl. 1 N NaOH was then mixed with the residue and allowed to stand at room temperature (23° to 25° C.) for 1 hour. The mixture was chilled, and 10 μl. of 6 M or 6.5 M perchloric acid was added. After 5 minutes the preparation was centrifuged, the supernatant fluid removed, and the residue extracted twice with 50-μl. volumes of 0.3 M perchloric

acid. These 3 extracts were combined as the *pentose nucleic acid* (PNA) fraction and brought to 200 μl. with 0.3 M perchloric acid. The optical density at 260 mμ was determined, and the value was used to calculate the quantity of pentose nucleic acid. The residue from the PNA extraction was treated with 50 μl. of 1.6 M perchloric acid at 60° C. for 7 minutes, cooled immediately in ice, centrifuged, and the supernatant fluid removed. The pellet was re-extracted twice with 0.3 M perchloric acid, and the 3 extracts were combined as the *deoxypentose nucleic acid* (DNA) fraction. This was made up to 200 μl. with 0.3 M perchloric acid and the optical density determined. The nucleic acid standards were commercial preparations which had been precipitated with hydrochloric acid and washed with ethanol and ether.

The tubes containing the *lipide* extract were carefully filled with water, immersed in a beaker of water at 2° C., and left overnight. The water in the beaker was taken off by suction and that in the tubes partially removed in the same way. The tubes were then centrifuged to complete separation of the phases and to pack the fluffy layer. Most of the remaining upper phase was removed through a pipette which had a very fine tip bent at a right angle. The *fluff* at the interface was brought into solution by the addition of methanol, and the fluid was evaporated in a desiccator over silica gel and paraffin chips under moderate vacuum after flushing with nitrogen. When the tubes seemed dry, the desiccator was evacuated with a rotary oil-sealed pump. After removal from the vacuum, the residue in the tubes was extracted 3 times with chloroform-methanol at room temperature (with centrifugation of the tubes each time), and the extracts were combined on tared 6-mm. pans punched from aluminum foil. The pans were sometimes warmed on a hot plate and placed under a stream of nitrogen to facilitate the drying of each extract. The pans were dried *in vacuo* and weighed on a quartz helix balance with a sensitivity of 1 mm. per 10 μg. The extension was estimated by eye to 0.1 mm. with a mirror-backed scale.

Dry weights were measured by pipetting aliquots onto tared aluminum foil pans with a siliconed pipette (GE Dri-film SC-87). The pans were dried *in vacuo* (rotary oil-sealed pump) over silica gel and weighed on the quartz helix balance. Solutions of potassium chloride and of tris(hydroxymethyl)aminomethane (primary standard grade, Sigma Chemical Co., St. Louis, Mo.) were used to calibrate the balance.

PNA was also estimated by Lusena's modification of the orcinol method adapted to small volumes (*20*). The test solution and enough water to make 100 μl. were placed in a 6 × 50-mm. culture tube and 100 μl. of 1 percent orcinol (recrystallized) in 0.1 percent $FeCl_3$ in concentrated HCl added. The fluid was centrifuged to the bottom, and the tube sealed by heating in an oxygen-gas flame and pinching with hot forceps. After cooling, the contents were mixed and the tubes immersed in boiling water for 60 minutes. After cooling in tap water, the tubes were opened and the optical density of the contents determined at 665 mμ. The optical density was about 0.8 for 1 μg. ribonucleic acid (RNA).

Burton's (*21*) modification of the diphenylamine procedure for DNA was adapted to the same volume scale, and the tubes were sealed, by pinching while hot, for the overnight incubation. The optical density was about 0.5 for 1μg. DNA.

Spectrophotometric studies were made in a Beckman spectrophotometer, model DU, equipped with capillary absorption cells of 5-cm. light path and 2-mm. bore [Kirk, Rosenfels, & Hanahan (*22*)], and a photomultiplier accessory.

Paper electrophoresis was carried out in horizontal strips of Whatman #1 filter paper, 1-inch wide, supported on a hollow brass box cooled with circulating water at 2° C. The paper was insulated from the brass with a sheet of "Parafilm" (Marathon Corp., Menasha, Wis.), which appeared to be an adequate electrical insulator. A Plexiglass cover was supported by spacers of rolled Parafilm along the sides to hold it 2 mm. above the paper. Parafilm covered the ends of the migration chamber and the ends of the paper strip down to the buffer. Power was supplied from a regulated source through silver-silver chloride electrodes in beakers of 0.1 N potassium chloride. These were connected to the buffer vessels with 0.1 N potassium chloride—3 percent agar bridges.

At the end of the run, the ends of the paper were torn off, and water at 35° to 38° C. was circulated through the brass box to dry the paper. Drying was completed in an oven. Protein was stained with ethanolic bromophenol blue after a prerinse with ethanol, and the excess dye was removed with 3 rinses in 5 percent acetic acid.

Counts of virus particles were made by sedimentation onto an agar surface (*23*), followed by fixation with osmium tetroxide vapor, preparation of a pseudoreplica, shadowing with chromium, and examination with the electron microscope.[4]

RESULTS

The findings in the analyses described are listed in table 1. Except as noted, each value cited was the average of 2 or more determinations on each sample indicated. With one exception, each virus preparation was derived from a single bird. Preparation PNAF was a pool of virus from 4 chickens used for comparison of 3 different methods for PNA determination. The PNA values given in table 1 were obtained by the ultraviolet absorption procedure.

From the means of the individual values, the myeloblastosis virus contains 9.9 percent nitrogen, 1.27 percent phosphorus, 2.17 percent PNA, and 35 percent lipide. Although variation was seen from one specimen to another, the range with the different samples was little, if any, greater than the range of the differences with the material from a single bird. This included also the estimates of nitrogen which would

[4] The authors are grateful to Dr. D. G. Sharp for the counts of the virus particles.

TABLE 1.—Chemical composition of the avian myeloblastosis virus

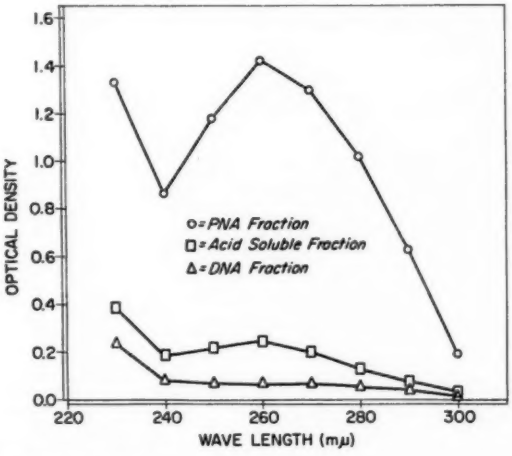
Medium	Preparation No.	Dry weight (μg.)	Total nitrogen		Total phosphorus		Pentose nucleic acid		Lipide	
			μg.	Percent	μg./μg. N	Percent	μg./μg. N	Percent	μg./μg. N	Percent
Ca-free Ringer's solution	A285		117		0. 140		0. 199		3. 8	
	P961		232		0. 129		0. 188		3. 0	
	P942		307		0. 134		0. 186		4. 2	
	A70		16		0. 116		0. 204			
Water	G918	2420	231	9. 6	0. 116	1. 18	0. 227	2. 17	3. 6	35*
	A61	1260	126	10. 0	0. 135	1. 34	0. 224	2. 23	3. 7	37
	A235	362	35	9. 7	0. 134	1. 31	0. 225	2. 19	3. 4	33*
	A250	2290	225	9. 8	0. 127	1. 25	0. 213	2. 10	3. 1	31
	A166	497	51	10. 3			0. 220	2. 28	3. 4	35
	G123	1810						2. 13		
	G123R	1760						2. 16		31*
	PNAF	3990						2. 07		42*
	Mean			9. 9	0. 129	1. 27	0. 210	2. 17	3. 5	35
	Standard deviation			0. 3	0. 009	0. 07	0. 016	0. 07	0. 4	4

*Single determination.

be expected to be of greater precision than those of the other constituents.

A notable feature of the findings was the low content of nucleic acid and the evidence that all was of the PNA type. In the determination of the nucleic acid by ultraviolet absorption, the optical density at 260 mμ was converted to micrograms of PNA by multiplication with the factor 1.45 which was obtained by application of the digestion and acidification treatments to purified yeast nucleic acid. The factor, allowing for differences in volumes and light paths, was somewhat higher than that used by Scott *et al.* (*19*), who calculated their values from absorption by mononucleotides. The differences in values may reflect some residual hypochromicity after 1 hour of alkaline digestion. It is possible that the increase in optical density characteristic of hydrolysis may not be complete after 1 hour and that use of the mononucleotide absorption values would then give a conversion factor somewhat too low.

The values for optical density of the PNA fractions were corrected for turbidity by Fog's method (*24*) when the correction was significant. It ranged from zero to 10 percent. Optical densities from 320 to 500 mμ were used to calculate the corrections. It is noteworthy that the "acid soluble" fraction absorbed light in the ultraviolet region and showed a small peak at 260 mμ. Because of the small quantity of this material absorbing at 260 mμ, its nature has not been determined. Its quantitative relation to the PNA fraction is shown in text-figure 1.



TEXT-FIGURE 1.—Ultraviolet absorption spectra of the respective fractions of 100 μg. of myeloblastosis virus.

Confirmation of the low level of PNA concentration indicated by the use of the ultraviolet absorption procedure was obtained by estimates with the orcinol reaction and determination of phosphorus. All these procedures were applied to the PNA fraction, that is, the material rendered "acid soluble" by alkaline digestion after preliminary extraction of acid-

soluble and lipide fractions. It is seen in table 2 that the values obtained with the 3 methods were essentially identical. When the orcinol test was made with whole virus, a higher value was found, namely, 3.62 percent of the dry weight, as compared with 2.17 percent observed with the isolated PNA fraction. The additional orcinol color may have represented pentose nucleotide appearing in other fractions, such as the acid-soluble fraction or non-nucleic acid carbohydrate or both.

TABLE 2.—PNA content of the myeloblastosis virus determined by ultraviolet absorption, by phosphorus estimate, and by the orcinol reaction

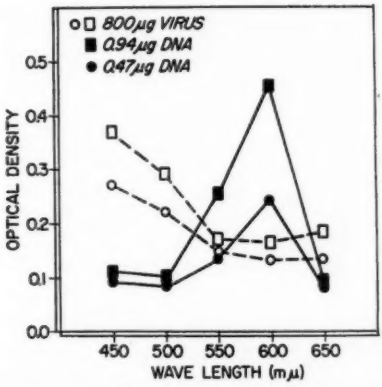
Sample	Ultraviolet absorption		Phosphorus determination		Orcinol reaction		Ratio of values	
	PNA*		PNA*		PNA*		P	Orcinol
	N	Percent	N	Percent	N	Percent	UV	UV
PNAF		2.07		2.15		2.14	1.04	1.03
P942	0.19		0.17				0.89	
A285	0.20		0.21				1.05	

*PNA/N=μg. PNA/μg. N.

To obtain further information as to whether the PNA found was an integral part of the virus, a sample of the purified agent was subjected to the action of ribonuclease. Virus was prepared from plasma G123 in the usual way except that the pellet obtained after 1 cycle of centrifugation was suspended in 0.14 M sodium chloride containing 0.01 sodium phosphate, *p*H 7.5, and then divided into 2 portions. One was treated with pancreatic ribonuclease (crystalline, Worthington) in 2 μg. per ml. concentration, and the other was left as a control. The samples were incubated for 20 minutes at 27° C., then chilled, and the centrifugal procedures for virus purification were continued as usual. The results obtained with the ribonuclease-treated virus preparation and the corresponding control are shown in table 1 as G123R and G123, respectively. There was no significant difference in PNA content with or without ribonuclease treatment. A control sample of yeast nucleic acid was rendered acid soluble by similar ribonuclease treatment.

DNA was absent as judged by the ultraviolet absorption procedure. To check this finding, the diphenylamine reaction was adapted to small volumes and applied to the whole virus. The result, as shown in text-figure 2, gave no indication of the presence of DNA in the agent. Most of the absorption by the virus suspension was probably due to turbidity. Even if all the absorption at 600 mμ were due to reaction of hypothetical viral DNA with diphenylamine, which it clearly was not, the DNA content would be only 0.04 percent of the dry weight of the agent. The ultraviolet absorption procedure indicates that DNA content could have been no greater than 0.1 percent.

Lipide was determined only as that material soluble from the desiccated state in chloroform-methanol without attempt at further fractionation.



TEXT-FIGURE 2.—Absorption spectra of the products of the diphenylamine reaction with whole myeloblastosis virus and with standard DNA.

The high concentration of phosphorus not bound in the nucleic acid suggests the probability that a large part of the lipide fraction was phospholipide. When the total lipide fraction was washed, a "fluff" appeared at the chloroform-water interface. When the washed lipide fraction was dried and extracted with chloroform-methanol, a small residue remained, which suggested the presence of a proteolipide type of complex.

The possible presence of plasma or other low molecular weight proteins in the virus preparations was investigated by means of paper electrophoresis. The buffers were 0.1 M sodium phosphate, *p*H 7.1; 0.1 M sodium diethylbarbiturate, *p*H 8.4; and 0.1 M sodium chloride—0.005 M tris(hydroxymethyl)aminomethane chloride, *p*H 7.0. The suspensions of the virus were subjected to electrophoresis at 3 V per cm., and the paper was stained with bromophenol blue. With this procedure there was no evidence of mobile protein. It was estimated that a single protein component of a few percent of the amount of the virus would have been detected if it had been present.

A comparison of the particle number, as determined by counting in the electron microscope, with the dry weight of the preparation is given in table 3. In the examination with the electron microscope, it was noted that the preparations in water remained fairly well dispersed even after

TABLE 3.—Direct determination of dry weight of myeloblastosis virus particles

Preparation No.	Particle No. (*per ml.* × 10^{10})	Dry weight (μg./ml.)	Mass per particle (μg. × 10^{10})
A61	140	1260	9.0
A235	61	362	5.9
A250	180*	2290	12.7
A166	65	497	7.6
	Mean of A61, A235, and A166		7.5

*This preparation was aggregated and counting was difficult.

several sedimentations, while preparations in calcium-free Ringer's solution were usually too aggregated for counting. A mean value of 7.5×10^{-10} μg. was found as the dry weight of a single virus particle.

DISCUSSION

A major consideration in the discussion of the present findings is that concerned with the state of homogeneity of the preparations under analysis. Unfortunately, because of properties inherent in the virus, the results obtained with the usual procedures for physical analysis do not furnish an accurate estimate of the state of homogeneity of the material. Thus, the virus particles vary in size, and preparations of them exhibit spreading, though single, boundaries in the analytical ultracentrifuge (*8*). Electrophoresis reveals single sharp boundaries, but preparations of the virus particles cause light scattering, and suspensions suitable for study can be only of low concentration. In work previously reported (*25*), it could be estimated that preparations of the purified agent perhaps contained no more than 25 percent of a material migrating in an electrical field at a rate different from that of the virus. The electrophoretic boundaries examined (*25*) gave no evidence of more than one component. Electron micrographs of virus sedimented on agar show (*25*) no evidence of a particulate component other than the virus. In the present work, care was taken to select only those plasmas of high virus concentration, namely, from about 10^{11} to more than 10^{12} virus particles per ml. It is known (*9*) that normal plasmas contain a population of nonviral particles of the order of 10^9 per ml., and no evidence has been seen that the number of such particles is greater in plasmas from diseased chickens than in those from normal birds. Thus, from the point of view of such nonspecific particles, it can be judged (*9*) that if the diseased plasmas contain as many as 10^{11} virus particles per ml., the product of ultracentrifugation can contain no more than perhaps 1 to 5 percent of nonviral particles. This is illustrated in figure 1, an electron micrograph of a thin section through a pellet of myeloblastosis virus sedimented directly from plasma. The possibility of significant serum protein contribution does not enter the problem, as was shown by electrophoresis in the present work and by earlier ultracentrifuge studies (*8*). It has not been possible to apply immunologic tests to determine contamination with normal host material, since the avian myeloblastosis virus has been shown to contain antigen indistinguishable from that of normal chicken tissue. Considerable evidence has been presented (*25*) to show that this normal host antigen is an integral part of the virus particle prepared from plasma.

Uniformity in chemical constitution is not, of itself, an acceptable criterion of homogeneity. However, when uniformity is encountered with a material known by other methods to consist, at least predominately, of virus, then constancy of composition assumes significance paralleling that of the findings with the other methods. In this virus, variation in

composition from one sample to another was no greater than that observed in multiple examinations of the same sample. Actually, when the methods employed and the fact that the minute samples came from different hosts are taken into consideration, it can be seen that the findings exhibit great uniformity. In this respect, it is evident that the results fully support previously obtained evidence of homogeneity and that they reflect accurately the chemical structure of the agent.

Of much importance is the evidence that, in its constitution, the agent, which induces directly a state of malignancy in the chicken, does not differ in physical and chemical attributes from viruses causing disease other than neoplasia. While the lipide content is somewhat high, it is less than that in the virus of equine encephalomyelitis. The concentration of nucleic acid is quite low but is of the order of that in the influenza virus (*27*). Some acid-soluble nucleotide may also be present in a small amount, possibly 0.1 percent of the dry weight of the virus. It may have been derived from the PNA during preparation for analysis, or from an unknown proportion of degraded virus. The preparations contain a small amount of material reacting in the orcinol test, in addition to that in the PNA fraction. It might be, in part, acid-soluble nucleotide, or nucleotide bound to proteolipide and soluble in organic solvents; nucleotide resistant to alkaline hydrolysis and hot perchloric acid; or non-nucleic-acid carbohydrate. Since the amount of this carbohydrate is small, about 0.5 percent of the dry weight of the virus (if it is pentose), further characterization will require relatively large amounts of purified agent. The absence of DNA is not exceptional. The content of phosphorus, in comparison with the nucleic acid, is very high, namely, about sixfold of what could be associated with the nucleic acid. It is probable that much of the excess of phosphorus will be identifiable with phospholipide.

The lack of DNA in the virus assumes importance, at the moment, in comparison with another virus associated with the occurrence of malignancy. This is the agent of rabbit papillomatosis which contains only DNA (*3*) in about 8 percent concentration. This is of particular interest in that the oncogenic viruses may very well be expected to vary quite as widely in constitution as other filterable agents which cause disease in man and animals. Furthermore, the difference suggests caution in speculation relative to the specific mechanisms of virus activity in the induction of various forms of recognized virus-induced malignancy.

Probably a significant observation in the study of ultrathin sections has been that the agents found in association with various tumors of the chicken, Rous sarcoma (*28*), Murray-Begg tumor (*29*), and the leukemias (*26, 30*), are similar in morphology. This has been the case with respect to size range and to characteristic distribution of elements within the particles staining with osmic acid. Thus, electron micrographs reveal the presence of a centrally placed "nucleoid" (*26*) of high electron absorbing power surrounded by material of low osmic acid reactivity. Recent work by Epstein and Holt indicates that PNA is an important constituent

of the nucleoid of the Rous virus (*31*). Because of the resemblance between the various chicken tumor agents and the evidence of immunologic relationship (*4*), it does not seem too remote to speculate that they may be similar in chemical constitution.

The particle weight of the virus may be estimated from the dry weight of a preparation and the electron microscope count of the number of particles. Use of the 3 more reliable values in table 3 gives a mean particle mass of 7.5×10^{-16} gm. Use of this mass and the value of 1.29 for the dry density obtained by centrifugation in D_2O (*8*) gives a particle diameter of 100 mμ assuming spherical shape. This is in fair agreement with estimates of particle diameter made by analytical centrifugation (*8*), and electron microscopic examination (*25*, *26*).

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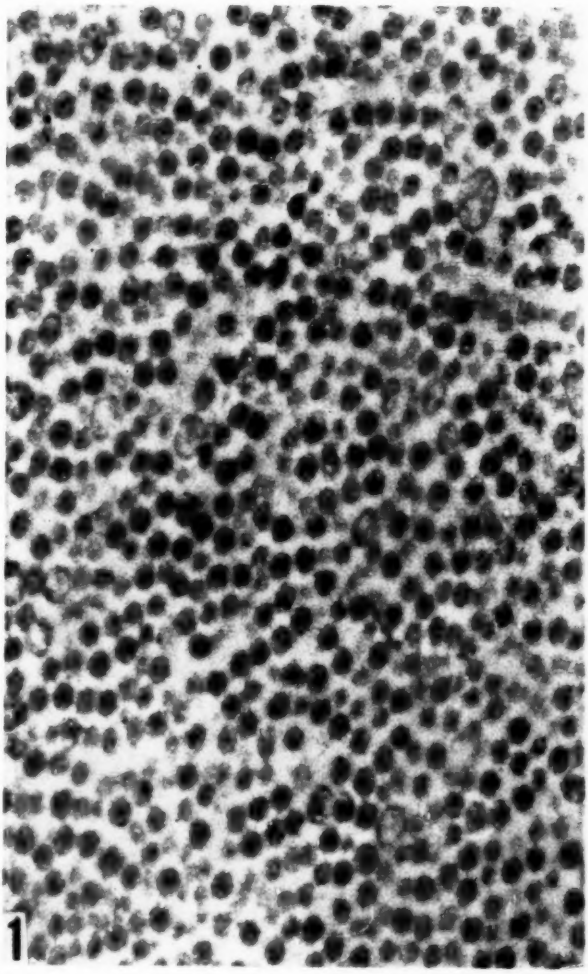
PLATE 14

FIGURE 1.—Electron micrograph of a thin section of myeloblastosis virus sedimented once from plasma of a bird with the disease. × 40,800

Courtesy Dr. W. Bernhard.



506956—59——22

Ultrastructure of Avian Myeloblasts in Tissue Culture [1,2,3]

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SUMMARY

Studies were made on the ultrastructure of avian myeloblasts from the circulating blood of chickens with myeloblastosis; on the cells after 2 days in tissue culture in 50 percent chicken serum; and on the cells after culture under the same conditions for 18 to 31 days. Brief examinations were made also of blast cells in the bone marrow of chickens without disease. Myeloblasts in the blood exhibited a structure indistinguishable from that of blast cells with the characteristics of myeloblasts in the bone marrow. The blood cells showed only infrequently any evidence of intracellular structure recognizable as related to virus infection and synthesis. Previous work had revealed some, but very little more, evidence of intracellular virus in the cells from cultures in serum of low, 20 percent, concentration. In contrast, cells placed in a high concentration of serum underwent a remarkable enhancement of activity and showed the presence of large numbers of "gray body" structures which contained identifiable virus particles. In cells from cultures carried 3 to 4 weeks there was a sequence of structures grading from gray bodies to vesicles and vacuoles containing virus particles in small numbers. In the overall studies a series of transitional forms was seen with apparent intercorrelations of gray bodies, mitochondria, vesicles, and vacuoles involved in virus synthesis and disposal. The cells multiplied rapidly, liberated virus at a high rate, and retained the characteristics of malignancy. It appears that the gray bodies and mitochondria are related structures and constitute the major site of virus synthesis.—J. Nat. Cancer Inst. 23: 199–225, 1959.

AVIAN MYELOBLASTOSIS (*1, 2*) is a highly malignant form of virus-induced leukemia in the chicken. The cell exhibiting the characteristics of malignancy is the myeloblast (*3*) which, in this disease, occurs in large numbers in the circulating blood (*4*). As shown in earlier studies (*5*), these primitive cells can be established and maintained for long periods in tissue culture in which the specific causative agent is continuously liberated from the cells. More recently, improvements have been made (*6*) in the conditions of culture so that the cells multiply at a rate permitting frequent divisions of the cultures, while maintaining a high rate of virus

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[2] This work was aided by research grants to Duke University from the American Cancer Society, Inc.; from the National Cancer Institute of the National Institutes of Health, Public Health Service; and by the Dorothy Beard Research Fund.

[3] Some of the results described here were reported at the Seventh International Cancer Congress, London, July 6-12, 1958.

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formation and liberation. Such cultures constitute an excellent system for definitive studies of the highly varied interrelationships of virus and host cells.

In previously reported investigations (*7*), examinations were made of the ultrastructure of myeloblasts, both those taken directly from the circulating blood and those held for a time in culture media of a low concentration of chicken serum. Further studies of this sort have been made of the cells cultured under the improved conditions. The results of the various studies have shown a remarkable spectrum of ultrastructural characteristics related apparently to the nutritional state of their environment. The present report describes the morphologic characteristics of these structures which seem to be associated with cell activity in the synthesis of the myeloblastosis virus.

MATERIALS AND METHODS

Myeloblasts were derived from chickens infected with myeloblastosis virus of the BAI strain A (*8*). During 10 years of study (*9*), this agent has consistently caused only one form of *leukemia*, and the primitive cells (myeloblasts) observed in the circulating blood of a given chicken were highly homogeneous with respect to type and morphologic characteristics.

The cells for culture were obtained from White Leghorn chickens of the inbred line 15 (*10*) that were inoculated intravenously with myeloblastosis virus in filtered plasma from a previous passage. Blood taken from the heart, with heparin, was centrifuged in the cold, and the cells from the thick upper layer of myeloblasts were pipetted off and resuspended in normal chicken serum. The cells were again sedimented, the wash serum was removed, and cultures were prepared in 5.0-ml. volumes, which contained 2.5 to 3×10^7 cells, penicillin, 50 units, and streptomycin, 50 μg. per ml. The individual cultures were established and handled, by the techniques previously described (*5*), in 50-ml. Erlenmeyer flasks closed with rubber stoppers, and incubated at 37° C. on a Gyrotory shaker. The medium consisted of chicken serum at 50 percent or greater concentration in medium 199.

For *thin sections*, circulating myeloblasts were obtained from the blood of chickens that had the disease, by heart puncture, with heparin as an anticoagulant. After low-speed centrifugation, primitive cells from the upper layer were taken up with fine-tipped, vaseline-coated pipettes and delivered in drops about 1 mm. in diameter directly into osmic acid solution. Cells from cultures were collected by centrifugation of appropriate volumes of culture fluid. A limited study was also made of normal chicken bone marrow. The procedures for the preparation of all specimens for thin-sectioning were essentially like those of Palade (*11*). The samples were fixed in the cold for 1 hour in a 1 percent osmic acid solution buffered to *p*H 7.4. After dehydration in alcohol and embedding

in methacrylate, the specimens were sectioned with a Porter-Blum microtome.

RESULTS

An examination was made of myeloblasts derived from three different environments: 1) directly from the circulating blood of diseased birds; 2) after a brief period of culture in 50 percent, or higher, concentration of serum; and 3) after intervals of 18 to 31 days of culture under the same conditions. Within the limits of the conditions of this study, the results confirmed the findings of the earlier work. In addition, however, potentialities of the myeloblasts under improved conditions of culture have been revealed, which were not apparent in the previously reported examinations.

Myeloblasts From the Circulating Blood

Extension of the study of myeloblasts fixed and sectioned immediately after removal from the circulating blood of chickens at the height of the disease has revealed no new principles. An outstanding feature of the findings with these cells, as already described (*7*), was the paucity of evidence of intracellular association of virus with the cells as they were taken from the circulation. Typical virus particles often were seen outside the cell, but in very limited numbers in vacuole-like structures inside the cell in only about 1 percent of the sections examined.

This finding was particularly impressive for several reasons. These cells, which occurred in large numbers in the circulation, are characteristic of the disease; they exhibit the behavior of malignant cellular elements in transmitting the disease by transplantation (*3*); and, because of their apparent initial derivation from virus-diseased hematopoietic tissue, there seemed good reason to expect them to show evidence of virus involvement. Furthermore, this finding contrasted greatly with the observation that great numbers of virus particles occurred in other elements, such as the reticular cells and, particularly, macrophages in the spleen and bone marrow in which the agent lay free in the cytoplasm and in well-bounded inclusion-like bodies. In these respects the myeloblasts differed also from the circulating erythroblasts of erythroblastosis in which virus has been identified as individual particles or as particles in inclusion-like structures in about 5 percent of the sections examined in this laboratory. Most confusing of all, however, was the knowledge (*5*) that such myeloblasts placed in tissue culture produced and released virus at a high rate.

The major features of the structure of the circulating myeloblast are illustrated in figure 1. These cells almost uniformly exhibited evidence suggestive of mild damage. The mitochondria appeared swollen and the cristae indistinct, whereas the endoplasmic reticulum (*12*) was little affected. Dilation of the perinuclear space and the occurrence of vacuoles

in the cytoplasm (occasionally containing virus particles) were frequently seen. Ribonucleoprotein granules, mainly lying free in the cytoplasm, were abundant but not as richly distributed as in tissue-culture cells. The nucleus showed a slight indentation occupied by the Golgi apparatus. The latter was indistinct in the circulating cells, and the centrioles were rarely seen. The nucleoplasm was abnormal for a primitive blast cell. Blast cells of the normal chicken bone marrow, in common with the majority of primitive cells, showed a fine, evenly distributed chromatin. In the circulating myeloblasts, there was a distinct loss of chromatin together with some clumping and margination. One or more nucleoli were present in about one third of the sections. However, in many instances, this structure was not clearly distinguished from clumped chromatin, especially, as in the chicken, it was not seen to adopt the skeinlike form found in human material. The nucleus did not show maturative asynchronism observed in some human acute leukemias.

The plasma membrane was usually intact and exhibited sheetlike or bullous processes in every section. The swollen, oval-shaped mitochondria were evenly distributed throughout the cytoplasm. The cristae were often indistinct and fragmented and bordered by a variable amount of very fine granular material. Occasionally the wall of the mitochondrion was ruptured, and similar fine material lay in the cytoplasm adjacent to it. In these areas the density of the ribonucleoprotein granules was reduced.

Endoplasmic reticulum (*12*) occurred most frequently as small vesicles scattered evenly throughout the cytoplasm. Less frequently it occurred as elongated sacs or long, double lamellae. Chicken myeloblasts, as well as normal lymphocytes, occasionally contained a structure identical with the compound vacuole in human leukocytes described by Low and Freeman (*13*).

A further study has been made of the "gray bodies" (previously described in *7*) which were round or oval structures with a single or double outer wall, intermediate in size between the vesicles of endoplasmic reticulum and mitochondria. They contained a fine granular material of variable, usually light, electron density and were usually grouped near the mitochondria. Identical structures were present, as indicated later, in cells of normal bone marrow. Occasionally internal structures reminiscent of cristae could be seen.

The more electron-dense bodies which resembled atypical fat vacuoles have also been previously described (*7*). It is possible that these represent early forms of granules.

In the studies of normal bone marrow, it was not possible unequivocally to identify individual cells as myeloblasts. However, a small percentage of the blast cells seen in the bone marrow exhibited a structure indistinguishable from that of the circulating myeloblasts of diseased chickens, as shown in figure 1. Of major significance in the examination of the bone marrow was the observation of typical structures like the gray bodies in those blast cells which resembled the cell of figure 1. Thus, it is evident that the gray body *per se*, though possibly involved in the processes

of virus synthesis, as described later, is, at its inception, a normal structure and not the result of virus infection.

Myeloblasts From Short-Term Cultures

In the earlier studies (7), examinations were made of cells held for varying periods in cultures containing 20 percent or less chicken serum. Under these conditions the structure, with few exceptions, was similar to that of the cells from the circulating blood. In some instances much virus was seen outside the cells. As before, very few cells were found to contain virus. However, a greater number of gray bodies was evident and, in these cells [fig. 2 of (7)] some of the gray bodies contained very small, highly osmophilic particles, with an appearance suggestive of incompletely formed virus. Although it was suspected that some relationship existed between the frequently demonstrable gray bodies and the more distinctive structures, which appeared as though they had viruslike particles included, the illustrations did not provide the basis for definite correlation.

In constrast, another appearance has been seen with cells in cultures containing chicken serum in 50 percent or higher concentration. The structure of myeloblasts in culture for 2 days is illustrated in figures 2 to 5. In general, the mitochondria were swollen, but the cristae were more distinct than in the circulating cells. The Golgi apparatus was well-developed, and the ergastoplasm retained its character of many free granules and relatively sparse reticulum. The appearance was quite uniform from one cell to another.

The most striking feature of the cells was the presence of a large number of structures not evident, as such, in cells from the circulating blood or in those from cultures containing 20 percent serum. Designated in the illustrations as GB, these structures were relatively highly osmophilic and varied widely in size and shape. Some were completely filled with the gray substance (figs. 2 and 4), while in others it was massed near the periphery, thinning and feathering toward an empty center (figs. 2 and 5). In most instances the bodies were enclosed by a single wall, but portions of a second inner wall were frequently seen.

In a large proportion of these bodies, embedded in the gray substance, were structures of the characteristic appearance of virus particles (figs. 2 to 5). In most instances in a given section of a given body, there were a few virus particles which were scattered through it. Close inspection of the bodies revealed a discontinuity of inner structure in many, which often appeared as fine lines that resembled remnants of mitochondrial cristae (fig. 5). Of particular interest was the occurrence (IF, figs. 3, 4, and 5) of ill-defined circular images also embedded in the gray substance. These had a variable diameter that ranged in size up to that of the virus particle. In many of these circular images, there was only the outlining wall and no evidence of internal structure like that in the characteristic virus particle. Other images, however, gave the impression of characteris-

tic virus structure and size, but were lacking in density. In looking through many gray bodies, a sequence of images could be seen ranging from faint circles without internal structure to typical virus particles.

Cells in the early cultures contained few empty vacuoles, although some clear spaces were seen, as in figure 3. Now and then, one or more virus particles were present in small vacuoles. As noted previously, the internal structure of the mitochondria was often sharply defined, in spite of some swelling. Virus was often seen outside the cells (fig. 4) but rarely in vacuoles and never definitely free in the cell cytoplasm.

Myeloblasts From Older Cultures

The structure characteristic of myeloblasts held in culture for 2 to 4 weeks is shown in figures 7 and 8. With such cells the findings differed greatly from those described for circulating cells and cells of early culture. In general, the illustrations gave the appearance of great cellular activity, as indicated by numerous thin or voluminous protrusions of the external cell wall and the numerous, sharply defined mitochondria.

In these cells a variety of structures occurred of somewhat uncertain nature and interrelationships. Forms were regularly present (GB, fig. 7) which closely resembled the gray bodies seen in circulating myeloblasts and in the myeloblasts cultured in chicken serum of low concentration. No evidence of virus was seen in such bodies.

There was evidence, however, that certain of the structures comprised a sequence of forms which could be assumed to range from mitochondria to small, usually peripherally located, vacuoles, with some containing one or a few virus particles such as VP in figure 8. In figures 7 and 8, the numerous mitochondria of these cells exhibit a characteristic structure with sharply defined cristae. Figures 7 and 8 show (AM) structures that have the appearance of altered mitochondria in which there is evidence, particularly in figure 8, of an arrangement of internal material which consists of irregularly distributed linear elements resembling disorganized cristae. As possible sequential transitional structures of mitochondrial derivation were the large, gray bodylike forms (GM in fig. 7) containing a homogeneously distributed electron-absorbing material, which, nevertheless, frequently showed some traces of fragmented strands. A series of structures extended from these bodies ranging from some filled with rather dense, formless material to others containing diminishing amounts of amorphous substance and frequently enclosing one or more typical virus particles (*see* right, fig. 8). An apparently final stage of these progressive changes was seen in numerous large and small, almost empty vacuoles (V in figs. 7 and 8), in which there were often a few typical virus particles and smaller bodies with the appearance, perhaps, of the central part of a virus particle. The smallest vacuoles were often clear, peripherally located, and contained one or more virus particles (VP in fig. 6).

As noted before, structures which might be classified as gray bodies were numerous, highly varied in size, and with a variable internal consistency

as judged by electron density. These ranged from completely filled bodies to others with vacuolar characteristics.

A remarkable difference between cells of early culture in high concentrations of serum and those cultured for longer periods was the smaller number of identifiable virus particles in the latter. In contrast with the early cultures, virus was seen less often in the gray body but frequently in small numbers in vacuoles (figs. 7 and 8), and was not at all free in the cytoplasm. In many sections, virus was seen (fig. 8) in the vesicle-like advanced forms of gray bodies which contained scattered amorphous material. Occasionally it appeared that these vacuoles could open to the outside to discharge virus.

Circulating Erythroblasts

Preliminary studies analogous to those with myeloblasts have been made of the characteristics of erythroblasts in the circulating blood and in tissue culture. For purposes of comparison and discussion, figure 9 shows an erythroblast from the blood of a bird with the disease. As already described in detail by others (*14*, *15*), the cell shows accumulations of characteristic virus particles in bodies that have the appearance of altered mitochondria (AM), in inclusion-like structures (IB), in vacuoles (V), and lying in a mass of amorphous material not clearly bordered by a wall (VP). In this cell are also highly osmophilic structures which resemble the gray bodies (GB) of the myeloblasts. Such massive accumulations of virus are often seen in the macrophages and reticular cells of the spleen and bone marrow of birds with erythroblastosis or myeloblastosis, but are not evident as part of the disease in the myeloblast in the circulating blood or in tissue culture.

DISCUSSION

An obvious problem in the study of virus-induced tumors is the nature of the influence of virus on the cell and the point of application of that influence. An obstacle to progress in this area, in contrast to analogous problems with some other virus diseases, has been the lack of a virus-host cell system suitable and accessible for study. The results of the present work are of much significance not only in revealing some of the aspects of virus-myeloblast relationships but also in the indication of the usefulness of this cell-virus system maintained free of the total influences of the host. Recent work (*5*, *6*) has shown that the myeloblast is fully amenable to continuous growth and to the exhibition of an extremely high activity, in tissue culture, of those processes which are associated with response to the virus and with the enduring state of virus synthesis and liberation from the cell.

The results of studies on myeloblasts from the circulation and those maintained in a low, 20 percent or less, concentration of chicken serum

have been quite disappointing in (*a*) the small evidence of virus in the cells; (*b*) the few structures possibly related to cell response to the agent; and (*c*) any clear suggestion of the manner of virus synthesis which occurred by direct measurement of the amount of agent liberated from the cells. Examination of cells maintained under the newer conditions, together with other information (*16*), however, has shown that the characteristics of the cells in the earlier experiments were probably related to nutritional deficiencies of the environment within the chicken and in culture. For practical reasons, cells for culture and other studies are usually taken from birds in the late stages of disease when the concentration of primitive cells is very high. Although few metabolic studies have been made of chickens with the disease, it was recognized early (*17*) that major disturbances occurred, for example, in serum potassium and magnesium. More recently, a profound hypoglycemia was observed (*16*), which developed in the late stages of both myeloblastosis and erythroblastosis. On the basis of these observations, it may be assumed that other equally drastic changes may have taken place which resulted in the reduction of the total nutritional level of the blood plasma. That 20 percent chicken serum in a basic saline medium, such as Gey's salt solution, was likewise inadequate for full activity of the cells was clearly shown by the study of thin sections and the behavior of the cells with respect to their own growth and the high rate of virus liberation in the better medium. As already described briefly (*6*), and more fully in a report in preparation, myeloblasts placed in 50 percent serum exhibit after 2 to 4 days a rapid increase in number and a regular, continued release of virus at a high rate. Transfer of the cells from the deficient medium of the circulating plasma resulted rapidly in a dramatic increase in cell activity as seen in the 2-day cultures. It is evident that the different states of the cells in the cultures carried longer is related not to the *age* of the culture but to the attainment of an equilibrium between cell activity and the nutritional level of the medium. Thus, it would be expected that the response of the cell would be lessened or increased by appropriate *changes* in the environment.

Evidence in the present work indicated that certain aspects of host-virus relationships were involved with the cell and its response to the agent. This may be viewed as a sequence of processes resulting in (*a*) the continuous synthesis of virus and its constituents; (*b*) in liberation of the agent from the cell; and (*c*) in the continuing involvement of previously unaffected structures in the same cell. The observations suggest that a major structure in virus infection and synthesis is the mitochondrion. Although, from these studies, a connection between certain gray bodies, especially the smaller ones, and mitochondria could not be detected, there is the possibility that such gray bodies represent developmental forms of mitochondria. The gray bodies of the myeloblasts resemble, morphologically, and are probably analogous to "microbodies," which were regarded by Rouiller and Bernhard (*18*) as precursors of the mitochondria in liver cells in hepatic regeneration. In the present work there was occa-

sionally a suggestion of a double outer wall and of cristae in the gray bodies. A definite link between the larger gray bodies and the mitochondrion in the myeloblasts was the evidence of the involvement of both in the processes of virus infection and synthesis. For purposes of further investigation it seems justifiable to assume that gray bodies and mitochondria are related structures and that, at some stage of development, they are the principal site concerned with virus synthesis.

Evidence has indicated (*15*) that at least one center of erythroblastosis virus synthesis is the mitochondrion. It has been shown, also, that in these structures the agent accumulates in walled masses or inclusion bodies, as illustrated in figure 9. Virus is often seen in the cell cytoplasm, but whether this is due to degradation and rupture of the inclusion bodies or is a part of an orderly process for ridding the cell of virus cannot be decided. It is a matter of observation that myeloblasts do not accumulate masses of intracellular virus particles. Instead, it must be concluded that, contrary to the situation in reticular cells and macrophages in myeloblastosis and in similar cells and erythroblasts in erythroblastosis, the rate of release of virus from myeloblasts closely parallels rate of formation. Furthermore, the processes of virus synthesis and disposal bring no harm to the myeloblasts, which exhibit progressive growth at high levels in an adequate medium. Erythroblasts have not yet been successfully grown for long periods in tissue culture, where they soon degenerate, and this may be related, for the most part, to properties inherent in the cell. In addition, accumulations of intracellular virus appear to damage the erythroblast, whereas, in great contrast, it is the influence of the myeloblastosis virus that confers on the host cell the capacity for continuous growth in tissue culture and for maintenance of the malignant state.

The manner of the disposition of mature myeloblastosis virus is obscure. There seems no reason to doubt that synthesis is a self-limiting process in a given gray body. As noted previously, the substance of the gray body seems to be consumed so that vacuole-like structures remain. The simplest structure obviously associated with the process is the small clear vacuole containing one or a few virus particles. Conceivably these may arise either by absorption or budding of the larger vacuole-like structures to constitute the final means for transporting the virus to the cell periphery, where it is expelled to the outside. In contrast, it was supposed (*14*, *15*) that the erythroblastosis virus was expelled *through* the cell membrane. No evidence of this has been seen with myeloblasts, though obviously it may have been overlooked.

Another question of major importance is that of the mechanism for continual infection of new mitochondria or gray bodies. In the rapidly dividing cells in culture, it is likely that infectious material is distributed between the daughter cells. It is obvious, however, that some means is required for the continuous infection of previously unaffected cell structures. This could occur by intracellular transfer, possibly of intact virus particles, for which there is actually no evidence. An alternate, and probably more likely, mechanism suggested by observations with other agents

(*19*) is the possibility of induction of the infectious process through the medium of ribonucleic acid (*20*) derived from intracellular virus or other centers of viral synthesis and transported through the cytoplasm to new receptive sites.

In a recent report, Dmochowski *et al.* (*21*) have questioned the observation of the rare occurrence of virus in myeloblasts taken directly from the diseased chicken. These authors stated, with respect to the investigations at Duke, "This is at variance with the findings of this study, in which cells with described changes may be regarded as granuloblasts." In answer to this criticism, nowhere in their publication was any specific finding illustrated or related to a cell identified and designated as a myeloblast. The authors imply that their findings with myeloblasts were the same as those shown in association with their "tumorous" cells of the spleen and bone marrow. This interpretation has not been supported by any evidence introduced by these investigators. As reported before (*7*), virus is readily found in the amounts and distribution in reticular cells and macrophages of the spleen and bone marrow of chickens with myeloblastosis like that described by Dmochowski and his associates. This has certainly not been true with myeloblasts. It would appear that the illustrations in the present report are adequate to show the actual interrelationships of the intracellular virus to the myeloblast. It may be added that the designation by Dmochowski *et al.* of all cells containing virus in both myeloblastosis and erythroblastosis as "tumorous" is particularly misleading. Although myeloblasts certainly behave as malignant cells, there is no evidence whatever that reticular cells or macrophages, *per se*, exhibit any characteristics of malignancy. In myeloblastosis, the only demonstrably malignant cell is the myeloblast. Until some positive evidence is available to support it, the designation of all types of cells containing virus in myeloblastosis and erythroblastosis as "tumorous" is not only confusing and misleading, but entirely without basis.

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PLATE 15

FIGURE 1.—Electron micrograph of thin section of myeloblast from circulating blood of chicken with myeloblastosis. Four mitochondria of typical appearance for these cells are grouped to *left* of nucleus. Numerous gray bodies (GB) are scattered through cytoplasm. The ergastoplasm consists of scattered bits of rough-surfaced reticulum and many free granules. Two extracellular virus particles occur at *left*, but none is visible inside cell. The nature of dark body at *upper end* is unknown. The morphologic characteristics of this cell do not differ from those of cells believed to be myeloblasts in normal bone marrow. × 32,000

GB

PLATE 16

FIGURE 2.—Myeloblast from circulation after 2 days in tissue culture in medium containing 50 percent chicken serum. In general morphology cell does not differ from myeloblasts taken directly from circulation (fig. 1). Lower part of cell shows gray bodies (GB) of irregular shape containing amorphous material embedded in which there are virus particles (VP) of typical appearance. The deeply stained bodies were not identified. × 24,000

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PLATE 17

FIGURE 3.—A portion of cell of figure 2 at higher magnification to show more clearly relation of virus particles (VP) to substance of gray bodies (GB). Indistinct forms (IF) of the size and shape but atypical appearance of virus particles are present. Gray bodies show suggestions of double walls. × 44,000

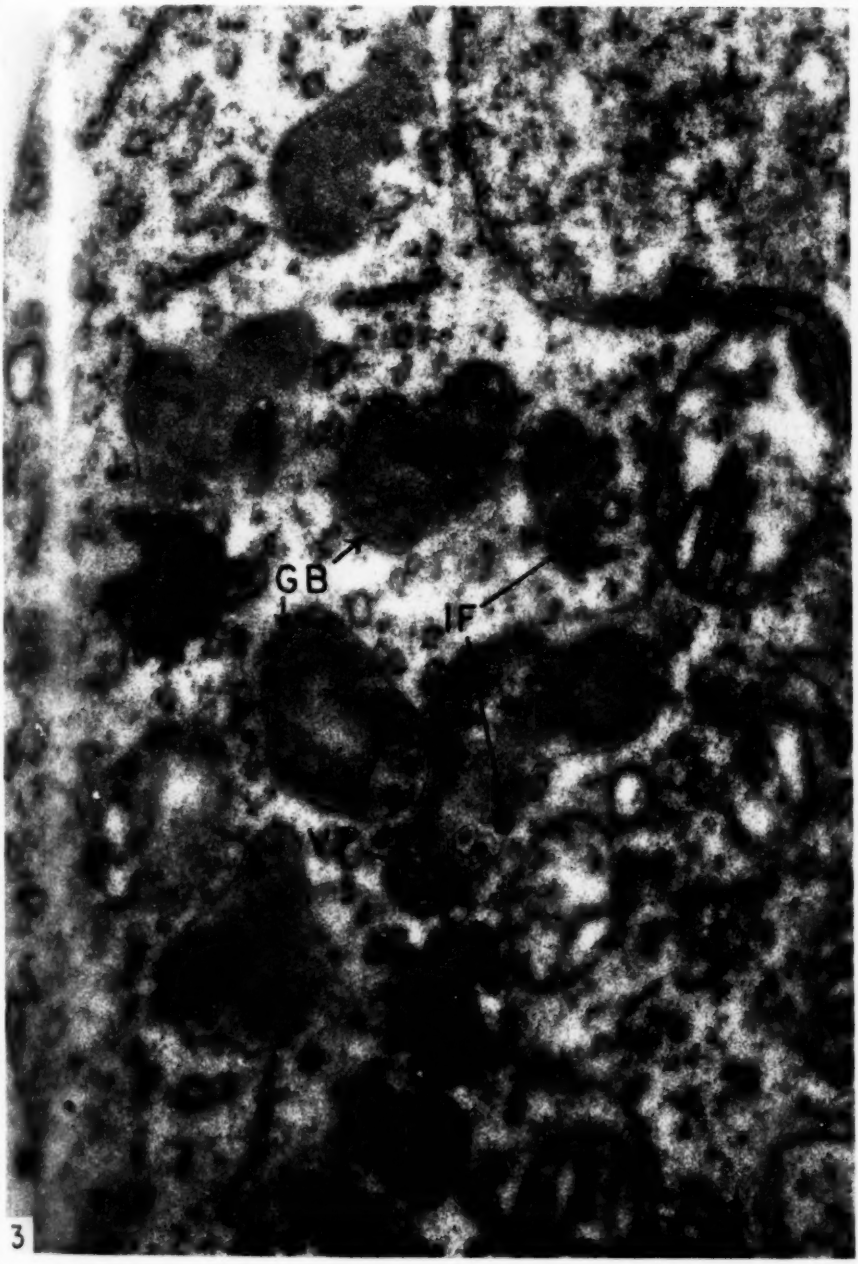

GB
IF
3

PLATE 18

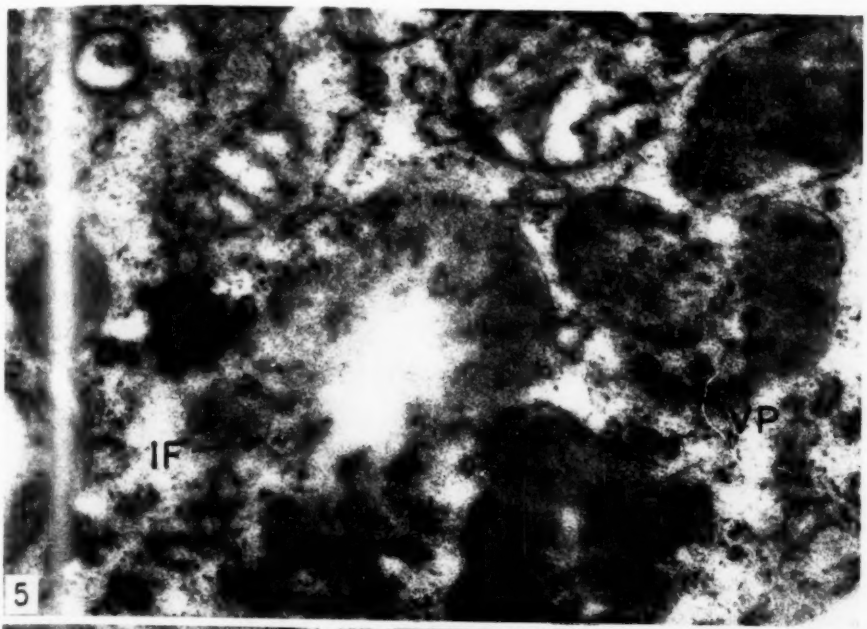
FIGURE 4.—Portion of another myeloblast from 2-day culture showing massive collection of gray bodies (GB) containing virus particles (VP) and indistinct atypical forms (IF). Mitochondria (M) are swollen, and the cell gives appearance of damage. × 46,000

4

PLATE 19

FIGURE 5.—High magnification of small part of myeloblast from 2-day culture to illustrate frequently seen gray bodies with open central areas. Typical virus particles (VP) are present as well as several indistinct forms (IF). × 52,000

FIGURE 6.—Small area of section of myeloblast from 17-day culture illustrating typical virus particles in peripheral vacuole (VP) and other particles outside cell at lower surface. × 71,000


IF
VP
5

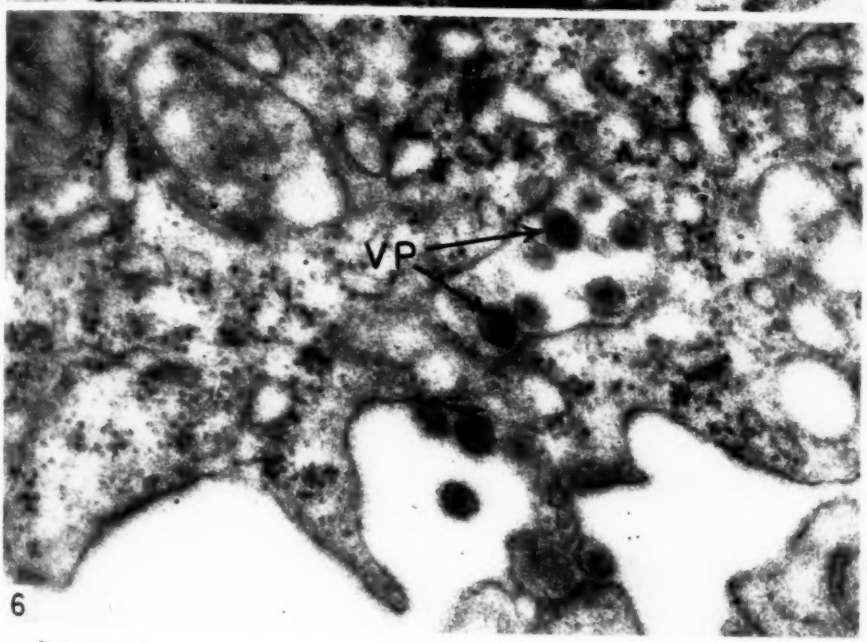

VP
6

PLATE 20

FIGURE 7.—Section of myeloblast, from 17-day culture in 50 percent chicken serum, cut to one side of nucleus. This illustration shows both normal structures and those associated with virus infection and synthesis characteristic of such cells. There are mitochondria (M) of well-defined structure; mitochondria in process of alteration (AM); gray bodies of mitochondrial derivation (GM); vacuoles (V) containing amorphous or particulate material; small gray bodies (GB); and a small peripheral vacuole containing virus particle (VP). There are numerous ribonucleoprotein granules; portions of Golgi apparatus (G); and of centrioles (C). Many virus particles are outside cell. × 27,000

BONAR *et al.*

PLATE 21

FIGURE 8.—Cell similar to that of figure 7 showing same sequence of structure but illustrating more clearly the transitional form of mitochondria (AM) containing disordered, disarranged cristae through gray bodies (GB), with varying amounts of amorphous inner material to almost clear vacuoles (V) containing virus particles (VP). × 39,000

8

PLATE 22

FIGURE 9.—Area of section of erythroblast from circulating blood of chicken with erythroblastosis. There are large numbers of virus particles (VP) packed in inclusion-like bodies (IB); a few in vacuoles (V); and one in structure with appearance of an altered mitochondrion (AM). Virus particles are outside cell at *lower left*. Some structures (GB) resemble gray bodies of myeloblasts. × 54,000

9

Announcement

First Directory of National Cancer Institute Research Fellows

The National Cancer Institute has just published the first directory of its research fellows. The fellowship program authorized by the National Cancer Institute Act of 1937 was the first of its kind to be supported by the Federal Government. It was initiated in a small way, but grew rapidly after increased appropriations became available for the Institute's work. Several hundred persons have now had the benefit of a period of training in cancer research, usually one to three years, as a result of receiving one of these fellowships.

Section I of the Directory contains the names of 924 persons whose fellowships had been completed by some date previous to April 1, 1958; Section II contains the names of 245 persons whose fellowships extended beyond that date. A brief statement about each fellow includes information as to the highest degree he held at the time of his appointment, the name of the school granting the degree, the type, duration, and place of his fellowship, and the nature of his research project. The present position of those whose fellowships had terminated by April 1 is also given.

The introduction to the Directory briefly discusses the program and points out that most of the former fellows are now engaged in research or teaching or both. About half of the fellows appointed in the early years of the Institute are now permanent members of the Institute's staff in key research positions. A number of the later fellows have also joined the staff.

Copies of the Directory have been sent to the libraries of appropriate institutions, including four-year colleges, medical schools, and foundations supporting research. The Directory is on sale by the Superintendent of Documents, Washington 25, D.C. Price per copy, 45 cents. A few copies are available free.

U. S. GOVERNMENT PRINTING OFFICE: 1959